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

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Supplementary information

Contents

A. Suggested experiment procedures

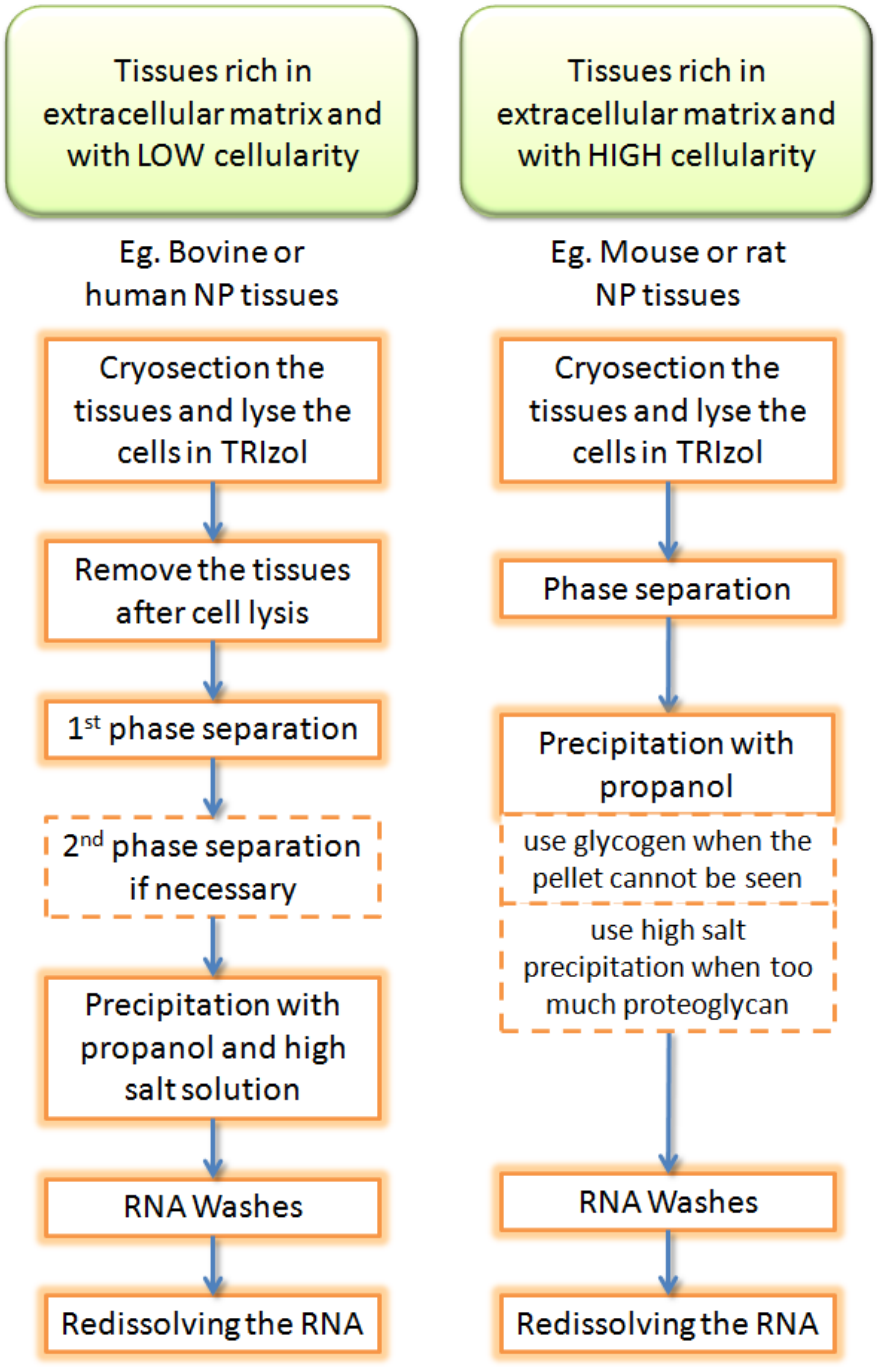

Figure S1. Suggestion of experiment procedures for extraction of RNA from tissues rich in extracellular matrix with low or high cellularity.

B. Experimental details of the comparison of RNA extracted using TRIzol and using combined TRIzol-column (TRIspin) method with cultured cells

Cells in wells of a 12 well plate (one well of bovine NP cells, one well of bovine AF cells and one well of bovine muscle cells) were lysed in TRIzol and TRIzol was added to a final volume of 5 ml. 300 µl of lysate was used for each sample for RNA extraction. The extraction of RNA using TRIzol was performed according to the manufacturers' instruction with the corresponding scaling of volume used. The RNA pellets were dissolved in 12 µl of DEPC-treated water. For the TRIspin method, the supernatant after first phase separation was mixed with 1/2 volume of ethanol and transferred to the RNeasy spin column. The subsequent procedures were performed according to the manufacturers' instruction. The RNA was eluted with 12 µl of DEPC-treated water and this step was repeated.

For the experiments with high proteoglycan content, cryosectioned NP tissue in a 1.5 ml tube (initially tissue volume of 100 - 250 mm^3) was lysed in 1 ml of TRIzol and mixed with cultured cells. TRIzol was added to a final volume of 5 ml. The subsequent procedures were same as those without high proteoglycan content.

Note: The combined TRIzol-column (TRIspin) method was based on a reported study by Wang *et al.* [1].

Simplified procedures in the two methods is given in the following diagram (Figure S2).

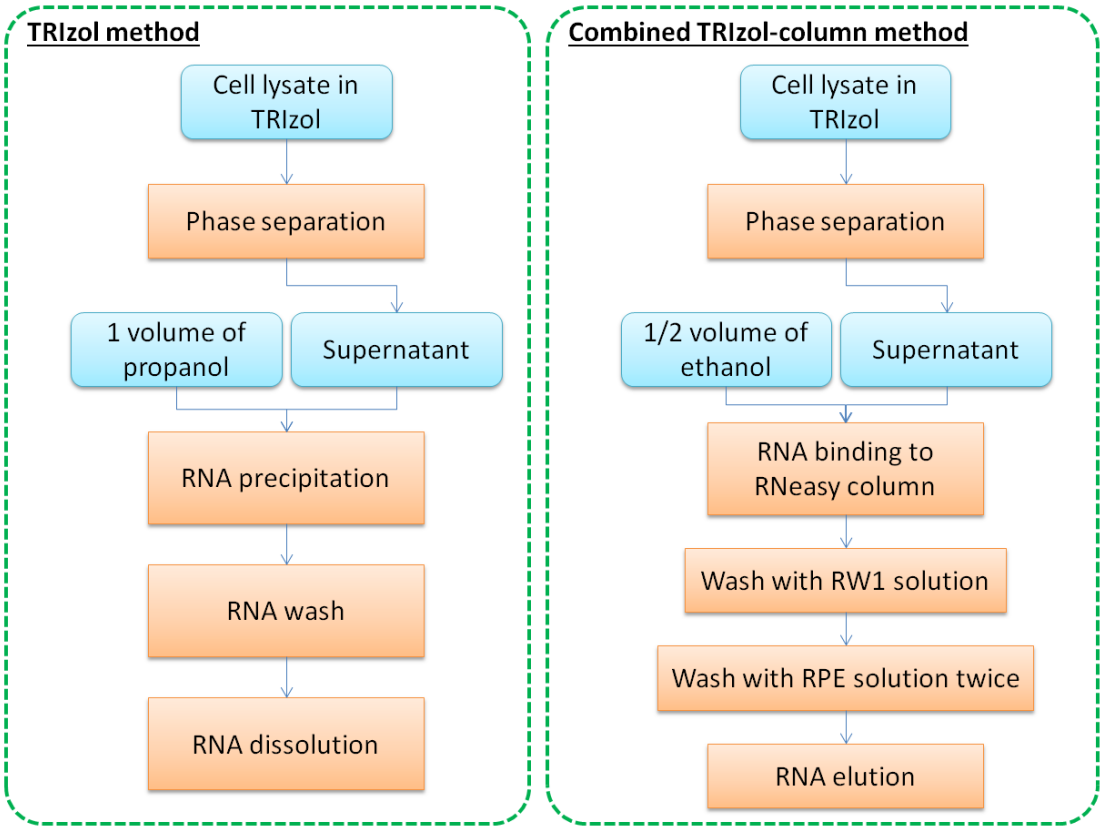

Figure S2. Simplified procedures in the TRIzol method and the combined TRIzol-column (TRIspin) method.

C. Recommended protocols for extracting RNA from bovine / human IVD tissues (tissues rich in extracellular matrix with low cellularity)

Cryosection of tissues and lysis of cells in TRIzol

1. Cryosection the tissues using a cryostat (10-30 µm)
2. Transfer the sections to 1.5 ml tubes (for a larger amount of tissue, the volume may be scaled up) using forceps pre-cooled in the cryostat chamber.
   Note: As the thin sections will melt and stick to surfaces at room temperature readily, it is important to pre-cool the tubes in the cryostat chamber. When holding the tubes, hold by the cap edges to avoid warming up the tube bodies.
3. Transfer the tubes of cryosectioned tissues to a fume hood using dry ice or Mr. Frosty pre-cooled to -80 °C (or any method that can avoid the thawing of the sections inside the tubes).
4. Add 0.5 ml of TRIzol to the tubes with samples, mix well, store at -80 °C before RNA extraction

1st phase separation

1. Centrifuge the samples and transfer the supernatant to new tubes so as to remove the tissues
2. Add 50 µl of bromochloropropane (BCP) to each tube with clear supernatant, shake vigorously for 15 s and stand at room temperature for 15 min
3. Centrifuge at 12,000 xg at 4 °C for 15 min

2nd phase separation (if necessary)

If the supernatant phase is milky, there is probably a high concentration of proteoglycan in it and it may be better to perform a second phase separation (see Figure 6A and 6B in the main text). If the supernatant phase is clear, it is better to avoid a second phase separation to avoid loss of RNA during the process.

1. Transfer the supernatant to new tubes
2. Add 1 ml of TRIzol and 150 µl of BCP. Shake vigorously for 15 s and stand at room temperature for 3 min
3. Centrifuge at 12,000 xg at 4 °C for 15 min
   Observation: The supernatant should become clear after the second phase separation (see Figure 6B in the main text).

Precipitation with propanol and high salt solution

1. Transfer the supernatant to new tubes and mix the supernatant with 0.3 ml of high salt solution (0.8M sodium citrate and 1.2M NaCl) and 0.3 ml of propanol (125 µl of high salt solution and 125µl of propanol if no 2nd phase separation was performed)
2. Stand at room temperature for 10 min and centrifuge at 15000 xg for 10 min

   Observation: pellet may not be easily observable at this step when the RNA quantity is low
3. Remove most of the supernatant using a 1 ml pipet tip and spin down the remaining supernatant again using a mini-centrifuge, then remove the supernatant using a 200 µl pipet tip as much as possible
   Note:
   - ✧ This is to remove the proteoglycan that is present in the supernatant which will precipitate later when 75% ethanol is added. When the supernatant is not completely removed, large pellets rich in proteoglycan may be resulted and entrap the RNA.
   - ✧ For working with precious samples for the first time, it may be better to save the supernatant in new tubes instead of discarding it just in case the RNA may be transferred accidentally with the supernatant.

RNA wash

1. Wash with 0.5 ml of 75% ethanol

   Observation: pellet (even though may be small) should appear at this step

2. Centrifuge at 8,000 xg at 4 °C for 3 min and remove the supernatant
3. Wash the pellet again with 0.5 ml of 75% ethanol
   Note: May extend the period of washing to get rid of the salts more completely. According to the manufacturer, the RNA can be stored in 75% ethanol at least 1 week at 4 °C.
4. Centrifuge and remove the supernatant
5. Dry at room temperature for 10 min

RNA solubilization

1. Add 12.5 µl of DEPC treated water and heat at 60 °C for 10 min
2. Cool on ice. Spin down, vortex briefly and spin down
3. Measure the absorbance using Nanodrop or assess the RNA quality and quantity through Bioanalyzer

D. Technical notes about using high salt solution and handling small pellets or precious samples

When using high salt solution

- ✧ When we precipitate RNA without the use of high salt solution, it is not important to remove the supernatant after the RNA precipitation step as much as possible. The supernatant remained will be miscible with the 75% ethanol to be added. However, when high salt solution is added, the miscibility with the 75% ethanol is changed. If too much supernatant (>50 µl) is left behind, there will be two different liquid phases after mixing with 75% ethanol in the form of a bubble in the liquid (Figure S3). The RNA will be on the surface of the bubble and the RNA cannot be easily washed as we need to get rid of the bubble with probably salts in it but avoid removing the RNA pellet. Thus the following precautions are required:
  - ■ Remove as much supernatant as possible after the RNA precipitation step when high salt solution is used. After removing the majority of supernatant using 1 ml pipet tips, use a second quick spin with a bench top centrifuge to bring all the liquid to the bottom and then remove the remaining liquid using 20 µl or 200 µl pipet tips.
  - ■ When a bubble of immiscible phase is already formed, try to remove the bubble using pipet tips with small openings such as 20 µl or 10 µl pipet tips to minimize the chance of sucking the pellet into the pipet tips.

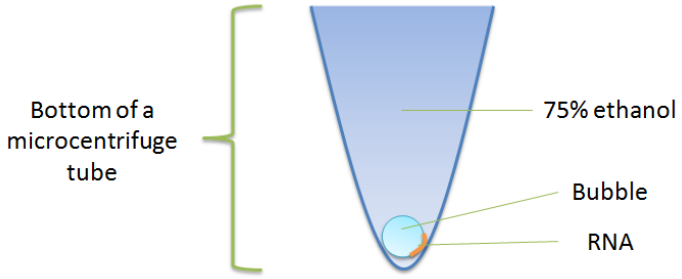

Figure S3. Schematic showing that a bubble may be formed after the 75% ethanol addition when the mixture of supernatant with propanol and high salt solution is not sufficiently removed

When handling small pellets

- ✧ Always make sure you can see the pellets and avoid discarding them during changes of solution, if not, add glycogen during the precipitation step so that

the RNA will be co-precipitated with the glycogen, making the pellets large enough to be observable.

✧ During the 75% ethanol wash, vortexing may be required for large pellets but not desirable for small pellets as this may break the pellets into smaller pieces which will increase the risk of discarding the RNA pellets during the 75% ethanol wash. Instead of vortexing, the RNA can be washed more efficiently by changing the orientation of tubes which can flip the RNA during the centrifugation as illustrated in Figure S4. Longer RNA wash duration may be used when necessary to remove the salts trapped in the pellets more effectively.

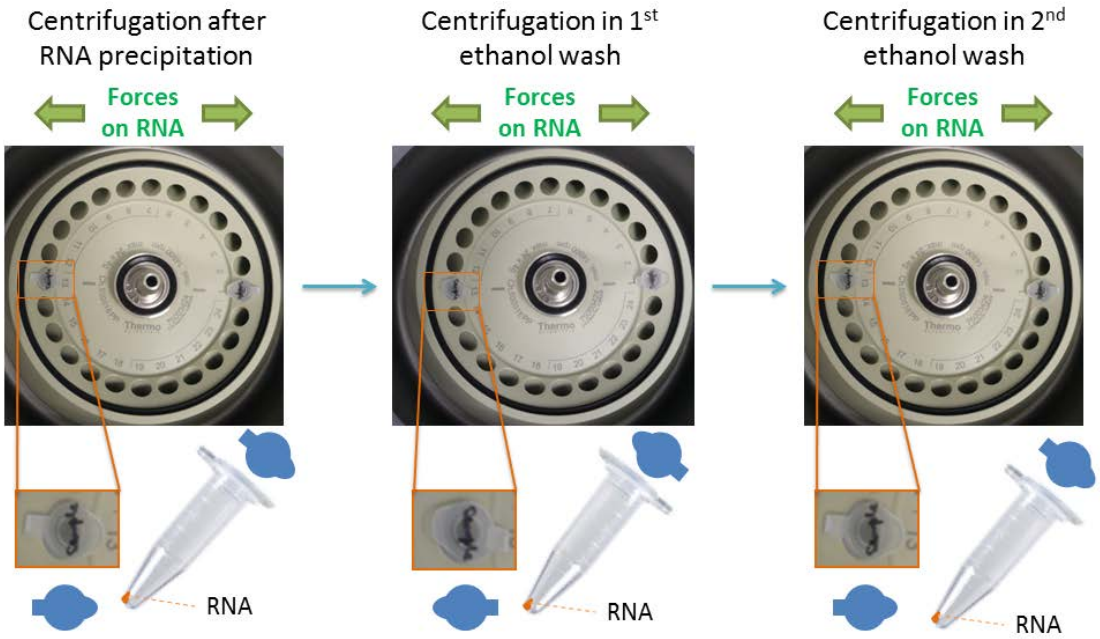

Figure S4. Schematic showing how the microcentrifuge tubes may be oriented during the centrifugation (1. The orientation of the tubes is oriented that it will be possible to know the position of pellets. 2. The orientation of the tubes is changed between successive centrifugation so that the pellet will be flipped on to the opposite side for more effective washing.)

When handling precious samples using the modified methods for the first time

If it is the first time to extract RNA using TRIzol from a small number of cells, test with one or two samples first and save the supernatant in extra tubes instead of discarding it. The RNA pellet can be rather small for a low RNA concentration and may be discarded accidentally with the supernatant for new learners of the TRIzol extraction. Besides, the RNA precipitation is likely to be less efficient when the RNA concentration is low. Thus it is better to save the supernatant phase after RNA

precipitation and centrifugation, especially when doing the extraction with precious samples for the first time, just in case there is RNA present in the supernatant not yet precipitated and centrifuged to the bottom of the tube.

E. Troubleshooting notes

Table S1. Troubleshooting notes for extracting RNA from tissues with high proteoglycan content with low cellularity. The following notes on troubleshooting are specific to samples with high proteoglycan content with low cellularity. Please also read the troubleshooting notes of the manufacturers when appropriate.

Problem	Possible causes and/ or *suggestions*
Large non-RNA precipitate upon precipitation	• The proteoglycan content is very high/ the supernatant is not sufficiently removed before RNA wash by 75% ethanol. o *Wash the RNA pellet with 75% ethanol thoroughly (eg. Extending the RNA wash duration since the large precipitate may trap salts more easily)* o *Re-extract the RNA with TRIzol if necessary*
Peak not at 260 nm	• The RNA concentration is too low / contaminant is present o *In such case, the concentration estimation of RNA may not be accurate.* o *Need to analyze the spectra further to determine the possible causes (Please refer to the troubleshooting notes of the manufacturer)*
Low A260/A230	• There may be salt contamination o *Re-extract the RNA with TRIzol (eg. When the pellet was large due to the presence of proteoglycan; the large pellet is more readily to trap salts). (Figure S5A and S5B)* o *Re-precipitate the RNA with ethanol and sodium acetate (Figure S5C and S5D)* o *For future similar extraction, increase the duration of RNA wash in 75% ethanol before drying and RNA dissolution (According to the manufacturer, the RNA can be stored in 75% ethanol at least 1 week at 4 °C.)*

Low RNA concentration	o *May pool the RNA from several samples to form one sample if possible and re-precipitate the RNA with ethanol and sodium acetate* o *May increase the centrifugation time after the RNA precipitation (but increasing the centrifugation time is not likely to help if there is already a pellet of more than 2 mm^3 observed)*
Peak at 270 nm	• It is likely that there is phenol contamination. In such case, the RNA concentration estimation is not accurate. The concentration can be corrected using a mathematical formula [2]. o *Further purification is required when the contamination level is high.*
Difficult to get rid of minute contamination (when the RNA concentration is low and glycogen is used as a co-precipitant or proteoglycan/ polysaccharide is present in the sample)	o *For RT-qPCR application, instead of adding the maximum volume of RNA for the reverse transcription, may be better to use a smaller proportion of RNA as the inhibition of the reactions is concentration dependent. Using a smaller proportion of RNA solution in the reaction may reduce the inhibition due to the contaminant.*

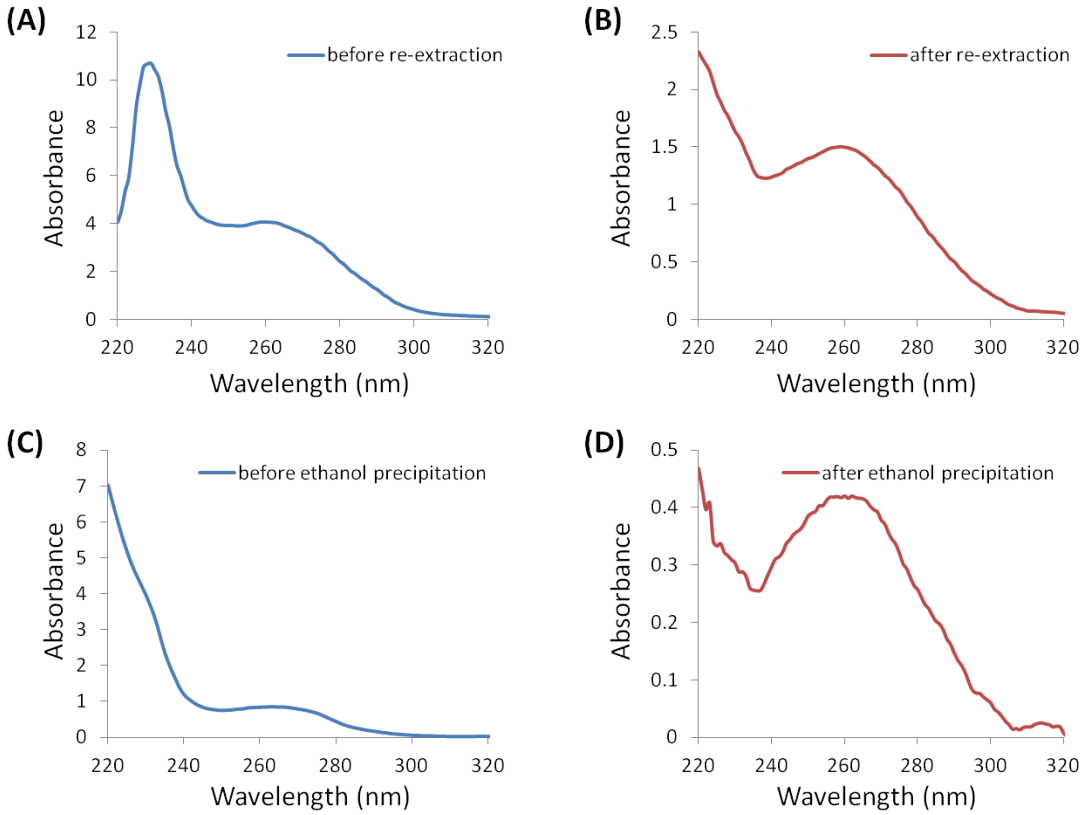

Figure S5. UV spectra of RNA (A) before and (B) after re-extraction with TRIzol; and (C) before and (D) after ethanol precipitation of RNA. Before re-extraction using TRIzol, there was a peak at 230 nm. The absorbance at 230 nm reduced after re-extraction. Similarly, ethanol precipitation of RNA reduced the absorbance at 230 nm. When the pellets are not large (< 2 µl), ethanol precipitation may be sufficient to remove the salts which contribute to the 230 nm absorbance. However, for large pellets with proteoglycan, it can be more difficult to remove the salts trapped in the pellets. In such case, re-extraction using TRIzol can be more effective as some of the proteoglycan will go into the organic phase during phase separation and get removed.

F. Recommended protocols for extracting RNA from mouse or rat IVD tissues (tissues rich in extracellular matrix but with relatively high cellularity)

Embedding of tissue in DEPC treated water

1. Prepare small aluminum containers of about 6 mm in diameter by using aluminum foil (cut it into appropriate size and form the container by wrapping around a pencil or the blunt end of a 2 ml serological pipet)
2. Add DEPC treated water to the container
3. Dip the container with water into liquid nitrogen using forceps (Handle with care when using liquid nitrogen) so that the periphery of the water is frozen while the centre remains in liquid state
4. Put the tissue in the top layer of water
5. Freeze the water with tissue completely by dipping it into liquid nitrogen using forceps
6. Remove the aluminum foil before mounting the ice block with tissue
7. Mount the ice block with tissue on the cryostat holder through the attachment of the surface opposite to the tissue

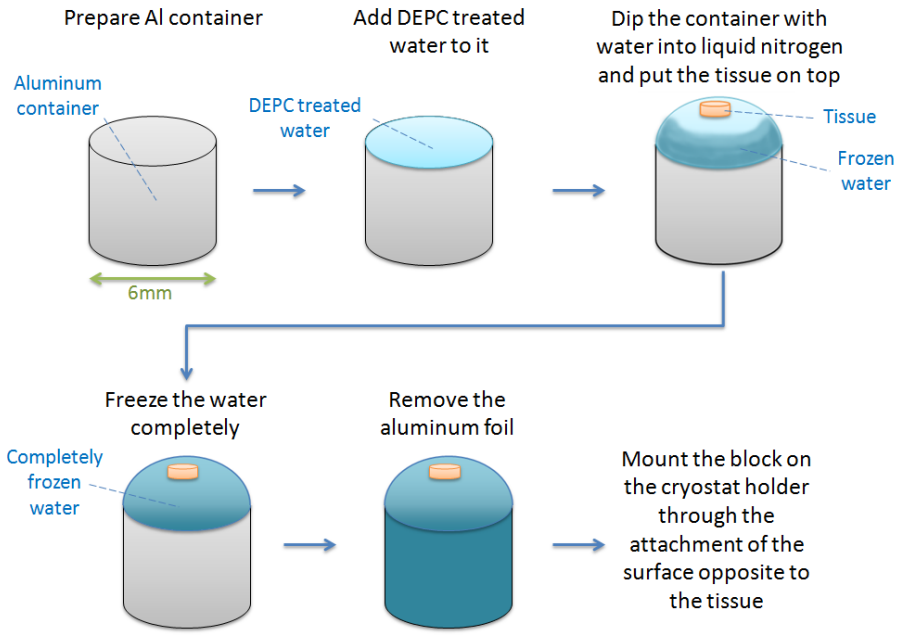

Figure S6. Schematic showing the procedures of embedding small tissue samples in DEPC-treated water prior to cryosectioning.

Cryosection of tissues and lysis of cells in TRIzol

1. Cryosection the tissues using a cryostat (10-30 µm)
2. Transfer the sections to 1.5 ml tubes (for a larger amount of tissue, the volume may be scaled up) using forceps pre-cooled in the cryostat chamber.
   Note: As the thin sections will melt and stick to surfaces at room temperature readily, it is important to pre-cool the tubes in the cryostat chamber. When holding the tubes, hold by the cap edges to avoid warming up the tube body.
3. Transfer the tubes of cryosectioned tissues to a fume hood using dry ice or Mr. Frosty pre-cooled to -80 °C (or any method that can avoid the thawing of the sections inside the tubes).
4. Add 0.5 ml of TRIzol to the tubes with samples, mix well, store at -80 °C before RNA extraction

Phase separation

1. Add 50 µl of bromochloropropane (BCP) to each tube with clear supernatant, shake vigorously for 15 s and stand at room temperature for 15 min
2. Centrifuge at 12,000 xg at 4 °C for 15 min

Precipitation with propanol and high salt solution

1. Transfer the supernatant to new tubes. Mix with equal volume of propanol (about 200-300 µl)
   Note
   - ✧ If there was a very large precipitate (>5 µl) for similar samples at the end of previous extraction, do the precipitation step with 125 µl of high salt solution (0.8M sodium citrate and 1.2M NaCl) and 125 µl of propanol.
   - ✧ If the RNA can hardly be seen after the precipitation and centrifugation, add 2 µl of 5 mg/ml glycogen to the sample, mix well and repeat the precipitation step
2. Stand at room temperature for 10 min and centrifuge at 15000 xg for 10 min

   Observation: pellet may not be easily observable at this step when the RNA quantity is low
3. Remove most of the supernatant using a 1 ml pipet tip and spin down the remaining supernatant again using a mini-centrifuge, then remove the supernatant using a 200 µl pipet tip as much as possible
   Note:
   - ✧ This is to remove the proteoglycan that is present in the supernatant which will precipitate later when 75% ethanol is added. When the supernatant is

not completely removed, large pellets rich in proteoglycan may be resulted and entrap the RNA.

- ✧ For working with precious samples for the first time, it may be better to save the supernatant in new tubes instead of discarding it just in case the RNA may be transferred accidentally with the supernatant.

RNA wash

1. Wash with 0.5 ml of 75% ethanol

   Observation: pellet (even though may be small) should appear at this step
2. Centrifuge at 8,000 xg at 4 °C for 3 min and remove the supernatant
3. Wash the pellet again with 0.5 ml of 75% ethanol
   Note: May extend the period of washing to get rid of the salt more completely. According to the manufacturer, the RNA can be stored in 75% ethanol at least 1 week at 4 °C.
4. Centrifuge and remove the supernatant
5. Dry at room temperature for 10 min

RNA solubilization

1. Add 12.5 µl of DEPC treated water and heat at 60 °C for 10 min
2. Cool on ice. Spin down, vortex briefly and spin down
3. Measure the absorbance using Nanodrop or assess the RNA quality and quantity through Bioanalyzer

G. qPCR amplification curves of mouse IVD and rat NP tissues

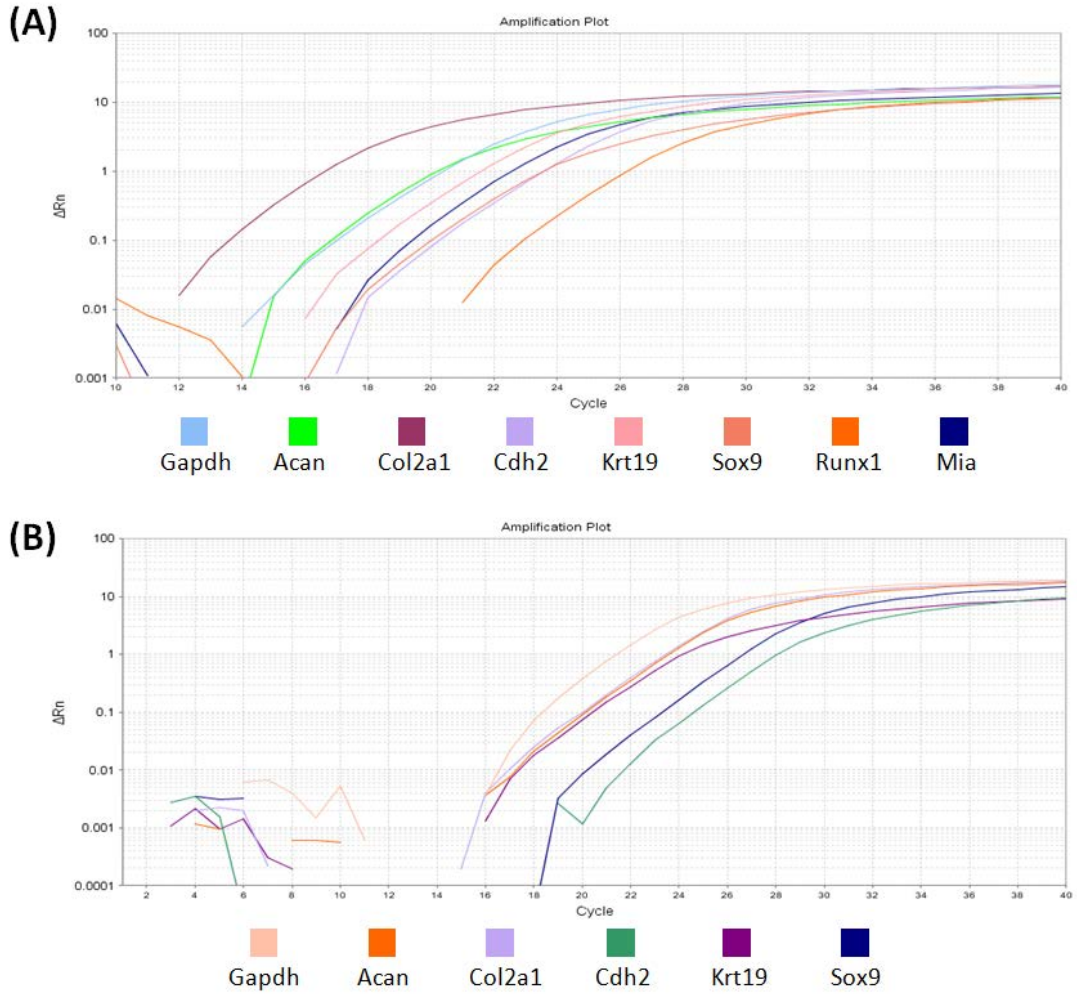

Figure S7. (A) qPCR amplification curves of RNA extracted from mouse IVD and (B) from rat NP.

H. Ct values of the RNA from bovine NP tissue extracted using TRIzol and TRIspin

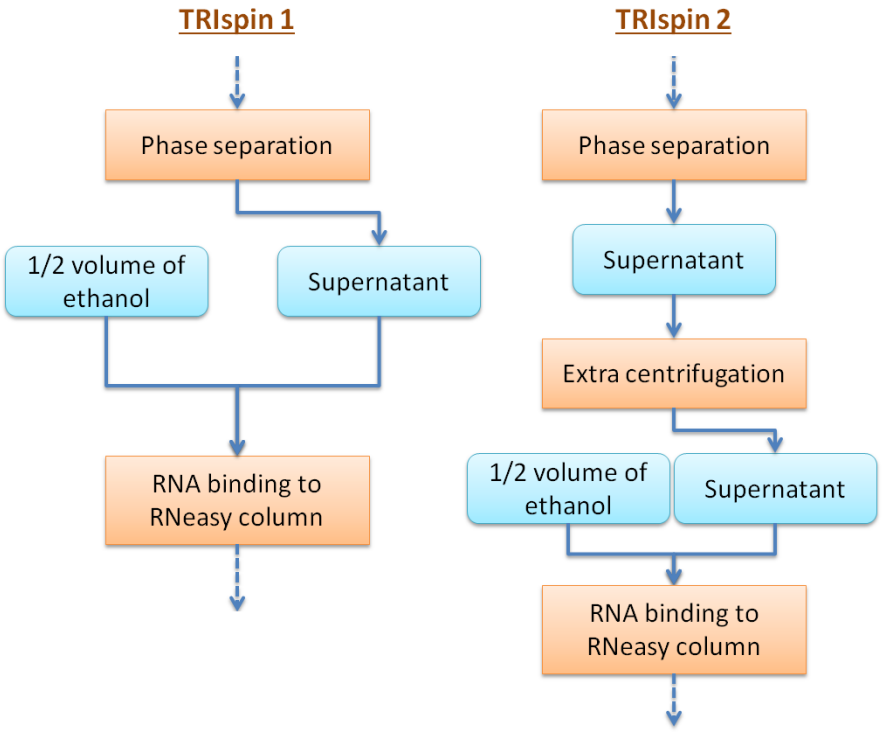

Figure S8. Simplified procedures for TRIspin 1 and TRIspin 2. Procedures prior to phase separation and after RNA binding to RNeasy column are not shown in the figure. For a comparison of the procedures of TRIzol and TRIspin, please refer to Figure S2 of this supplementary information file.

Table S2. Raw Ct values of the RNA extracted from bovine NP tissues using modified TRIzol or TRIspin after DNase1 treatment and RT-qPCR. [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 DNase1 treatment). [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. [c.] Only 1 out of the 3 samples has detectable amplification. (*GAPDH*, *18S rRNA*, *YWHAZ* are housekeeping genes and *ACAN* and *COL2A1* are matrix genes highly expressed in NP cells and chondrocytes) (mean ± SD; n=3)

	GAPDH	*18S*	*YWHAZ*	*ACAN*	*COL2A1*
TRIzol [a]	26.3 ± 2.2	17.6 ± 1.7	30.5 ± 2.5	23.3 ± 0.2	21.3 ± 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

Table S3. Adjusted Ct values of the RNA extracted from bovine NP tissues using modified TRIzol or TRIspin after DNase1 treatment and RT-qPCR. [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 DNase1 treatment). The Ct values were adjusted based on 10 ng of RNA used for DNase1 treatment for equal volume basis of starting materials. [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. [c.] Only 1 out of the 3 samples has detectable amplification. (*GAPDH*, *18S rRNA*, *YWHAZ* are housekeeping genes and *ACAN* and *COL2A1* are matrix genes highly expressed in nucleus pulposus cells and chondrocytes) (mean ± SD; n=3) (*GAPDH*, *18S rRNA*, *YWHAZ* are housekeeping genes and *ACAN* and *COL2A1* are matrix genes highly expressed in NP cells and chondrocytes)

	GAPDH	*18S*	*YWHAZ*	*ACAN*	*COL2A1*
TRIzol [a]	25.7 ± 1.0	17.0 ± 0.7	29.9 ± 1.3	22.7 ± 1.1	20.7 ± 0.7
TRIspin 1 [a, b]	31.0 ± 2.0	23.5 ± 1.6	31.0 [c]	29.4 ± 0.1	26.3 ± 1.1
TRIspin 2 [a, b]	30.5 ± 1.9	22.9 ± 1.6	Undetectable	29.0 ± 0.9	26.4 ± 1.5

I. UV spectra of RNA extracted with the TRIspin method without an extra centrifugation of supernatant

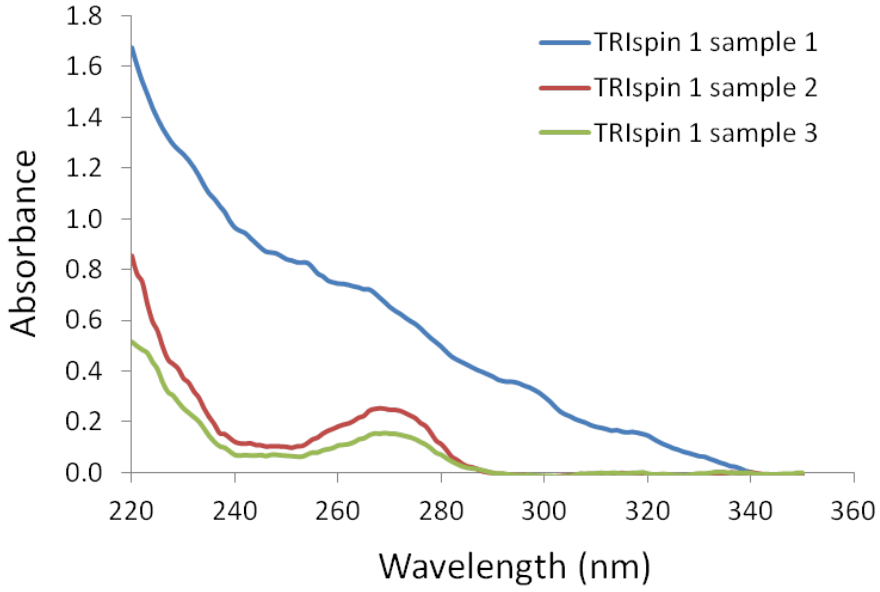

Figure S9. UV spectra of RNA extracted with the TRIspin method without an extra centrifugation of supernatant after TRIzol phase separation before ethanol addition. One out of the three samples without an extra centrifugation showed a UV absorbance curve without any peak in the 220-350 nm range, which may be due to proteoglycan contamination.

References

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2. Lee JTY, Cheung KMC, Leung VYL (2014) Correction for concentration overestimation of nucleic acids with phenol. Anal Biochem 465: 179-186.