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Quantitative real-time PCR evaluation of microRNA expressions in mouse kidney with unilateral ureteral obstruction

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Corresponding Author:	Katsunori Yanai Jichi Ika Daigaku Fuzoku Saitama Iryo Center Saitama City, Saitama Prefecture JAPAN
Corresponding Author's Institution:	Jichi Ika Daigaku Fuzoku Saitama Iryo Center
Corresponding Author E-Mail:	yanai03310751@yahoo.co.jp
Order of Authors:	Katsunori Yanai
	Yoshiyuki Morishita
	Shohei Kaneko
	Hiroki Ishii
	Akinori Aomatsu
	Kiyonori Ito
	Keiji Hirai
	Susumu Ookawara
	Kenichi Ishibashi
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TITLE:

Quantitative Real-Time PCR Evaluation of microRNA Expressions in Mouse Kidney with Unilateral Ureteral Obstruction

AUTHORS & AFFILIATIONS:

Katsunori Yanai[1], Yoshiyuki Morishita[1]*, Shohei Kaneko[1], Hiroki Ishii[1], Akinori Aomatsu[1,2], Kiyonori Ito[1], Keiji Hirai[1], Susumu Ookawara[1], Kenichi Ishibashi[3]

[1]Division of Nephrology, First Department of Integrated Medicine, Saitama Medical Center, Jichi Medical University, Saitama, Japan

[2]Division of Intensive Care Unit, First Department of Integrated Medicine, Saitama Medical Center, Jichi Medical University, Saitama, Japan

[3]Department of Medical Physiology, Meiji Pharmaceutical University, Tokyo, Japan

Email addresses of co-authors:

Katsunori Yanai	yanai03310751@yahoo.co.jp
Shohei Kaneko	shohei.sasurai@gmail.com
Hiroki Ishii	hi.ro.ki.i.99@gmail.com
Akinori Aomatsu	ayksera@gmail.com
Kiyonori Ito	kiyonori.ito@gmail.com
Keiji Hirai	keijihirai@kfy.biglobe.ne.jp
Susumu Ookawara	su-ooka@hb.tp1.jp
Kenichi Ishibashi	kishiba@my-pharm.ac.jp

***Corresponding author:**

Yoshiyuki Morishita

ymori@jichi.ac.jp

KEYWORDS:

molecular biology, microRNA, complementary DNA, unilateral ureteral obstruction, kidney, renal interstitial fibrosis, qRT-PCR

SUMMARY:

We describe a method for evaluating the microRNA expression in the kidneys of mice with unilateral ureteral obstruction (UUO) by quantitative reverse-transcription polymerase chain reaction. This protocol is suitable for studying kidney microRNA expression profiles in mice with UUO and in the context of other pathological conditions.

ABSTRACT:

MicroRNAs (miRNAs) are single stranded, non-coding RNA molecules that typically regulate gene expression at the post-transcriptional level by binding to partially complementary target sites in the 3' untranslated region (UTR) of messenger RNA (mRNA), which reduces the mRNA's translation and stability. The miRNA expression profiles in various organs and tissues of mice have been investigated, but standard methods for the purification and quantification of miRNA

in mouse kidney have not been available. We have established an effective and reliable method for extracting and evaluating miRNA expression in mouse kidney with renal interstitial fibrosis by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The protocol required five steps: (1) creation of sham and unilateral ureteral obstruction (UUO) mice; (2) extraction of kidney samples from the UUO mice; (3) extraction of total RNA, which includes miRNA, from the kidney samples; (4) complementary DNA (cDNA) synthesis with reverse transcription from miRNA; and (5) qRT-PCR using the cDNA. Using this protocol, we successfully confirmed that compared to the controls, the expression of miRNA-3070-3p was significantly increased and those of miRNA-7218-5p and miRNA-7219-5p were significantly decreased in the kidneys of a mouse model of renal interstitial fibrosis. This protocol can be used to determine the miRNA expression in the kidneys of mice with UUO.

INTRODUCTION:

MicroRNAs (miRNAs) — the short, noncoding RNAs that cause the degradation and transcriptional inhibition of messenger RNA (mRNA)[1] — have been shown to regulate the expression of various mRNAs that have crucial roles in both physiology and disease (e.g., inflammation, fibrosis, metabolic disorders, and cancer). Some of the miRNAs may therefore be candidate novel biomarkers and therapeutic targets for a variety of diseases[2-5]. Although miRNA expression profiles in mouse organs and tissues including brain, heart, lung, liver, and kidney have been described[6-10], there have been no standard methods for the extraction and evaluation of miRNAs in mouse kidney with renal interstitial fibrosis.

We have designed a protocol to reliably purify and detect the expressions of miRNAs in the kidneys of mice with renal interstitial fibrosis. The protocol involves five main steps, as follows. (1) 8-week-old C57BL/6 male mice are divided into groups of mice that undergo a sham-operation (controls) and mice that are subjected to a surgery providing unilateral ureteral obstruction (UUO), which is linked to renal interstitial fibrosis. (2) Kidney samples are extracted from the sham and UUO mice, homogenized separately in a silicon homogenizer, and then transferred to a biopolymer-shredding system on a microcentrifuge spin column[11,12]. (3) The total RNA containing miRNA is extracted from the kidney samples by a silica membrane-based spin column[12,13]. (4) Using this extracted total RNA, complementary DNA (cDNA) is synthesized from the total RNA with the use of reverse transcriptase, poly(A) polymerase, and oligo-dT primer[14,15]. (5) The expressions of miRNAs are evaluated by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using an intercalating dye[14,15].

This protocol is based on investigations that obtained meaningful extractions and evaluations of miRNAs in a variety of tissues[11-15], and the biopolymer-shredding system used in the protocol was shown to purify high-quality, total RNA from tissues in 2006[12]. In addition, prior studies have confirmed the accuracy and sensitivity of aspects of the protocol (i.e., the cDNA synthesis with reverse transcriptase, poly(A) polymerase, and oligo-dT primers from extracted total RNA) for the determination of miRNA expression by qRT-PCR with an intercalating dye[14,15]. Since the new protocol has the advantages of simplicity, time-saving, and the reduction of technical errors, the protocol can be used in research that requires the accurate and sensitive

identification of the miRNA profile in mouse kidney. Moreover, the protocol could be applied to investigations of many pathological conditions.

We next describe the determination of the miRNA expression profiles in mice with UUO, which is linked to renal interstitial fibrosis. In humans, renal interstitial fibrosis is a common and important feature of both chronic kidney disease and end-stage renal disease, regardless of their etiology[16,17]. This renal interstitial fibrosis is associated with the progression of renal failure, and it is characterized by increased expressions of extracellular matrix components in the interstitial spaces (e.g., collagen, fibronectin, and α-smooth muscle actin)[17,18].

PROTOCOL:

All animal experimental protocols were approved by the Animal Ethics Committee of Jichi Medical University and were performed in accordance with the Use and Care of Experimental Animals guidelines from the Jichi Medical University Guide for Laboratory Animals.

1. The sham surgery

1.1. Prepare the following items: isoflurane, cork sheet, depilatory cream, laboratory wipes, Petri dish with phosphate-buffered saline (PBS), 4-0 nylon, tweezers, surgical scissors, cotton swabs, and 8-week-old C57BL/6 male mice.

1.2. Anesthetize a mouse with 1.5% isoflurane and maintain at 1.5%. Then, apply depilatory cream to the mouse's abdomen. After a few minutes, wipe the depilatory cream off with a PBS-soaked laboratory wipe.

1.3. Inject 70% ethanol into the mouse's abdomen and then place the mouse on the cork sheet in the supine position.

1.4. Using surgical scissors and tweezers, make an incision in the skin at the abdomen and cut the muscle and peritoneal membrane from the bladder to the left lower edge of the ribs.

1.5. Moisten two cotton swabs with PBS and then pull the intestines carefully to the side. Place the moistened swabs to identify the left kidney and ureter.

1.6. Close the peritoneal membrane and then close the incision with 4-0 nylon.

2. The UUO surgery

2.1. Prepare the following items: isoflurane, cork sheet, depilatory cream, laboratory wipes, Petri dish with PBS, 4-0 silk, 4-0 nylon, a 2.5 mL syringe, cotton swabs, tweezers, surgical scissors, and 8-week-old C57BL/6 male mice.

2.2. Anesthetize a mouse with 1.5% isoflurane and maintain at 1.5%. Then apply depilatory cream to the mouse's abdomen. After a few minutes, wipe the depilatory cream off with a PBS-soaked laboratory wipe.

2.3. Inject 70% ethanol into the mouse's abdomen mouse and then place the mouse in the supine position on the cork sheet.

2.4. Using surgical scissors and tweezers, make an incision in the skin at the abdomen and cut the muscle and peritoneal membrane from the bladder to the left lower edge of the ribs.

2.5. Place the 2.5 mL syringe underneath the mouse. Take two cotton swabs and moisten them with PBS. Pull the intestines carefully to the side with the tweezers, and place the moistened swabs appropriately to identify the left ureter. Using the tweezers, lift the left kidney.

2.6. Use the 4-0 silk to ligate the left ureter in two places approx. 1 cm apart. Cut the ureter at the center point of the two ligations, and then use 4-0 nylon sutures to close the peritoneal membrane and incision.

3. Collection of kidney samples

3.1. Prepare the following: 1.5 mL microcentrifuge tubes, isoflurane, cork sheet, 70% ethanol, Petri dish with PBS, tweezers, and surgical scissors.

3.2. Anesthetize a mouse with 1.5% isoflurane and maintain at 1.5%. Inject 70% ethanol into its abdomen and put the mouse on the cork sheet in the supine position.

3.3. Using surgical scissors and tweezers, make an incision in the skin at the abdomen and cut the muscle and peritoneal membrane from the bladder to the left lower edge of the ribs.

3.4. Lift up the peritoneal membrane with the tweezers. With the surgical scissors, make a sideways incision at the upper edge of the peritoneal membrane, and continue the incision along the lowest edge of the ribs.

3.5. Next, identify the left kidney, reflux it with PBS until the kidney turns yellow-white to wash out blood from vessels, and remove the kidney by cutting the left renal artery and vein with the surgical scissors. Place the kidney in the Petri dish and wash it carefully with PBS.

3.6. Cut the kidney into 10 mg samples with the surgical scissors and tweezers (10 mg is an appropriate size for the next step). Put each piece of the kidney in its own 1.5 mL microcentrifuge tube and close the tube's cap.

3.7. Transfer each microcentrifuge tube into liquid nitrogen, and keep the tubes at −80 °C for long-term storage before use.

4. Extraction of total RNA from the kidney samples

4.1. Prepare the following items: 1.5 mL microcentrifuge tubes, 2.0 mL microcentrifuge tubes, 100% ethanol, chloroform, silicon homogenizer, ice, a vortex mixer, biopolymer spin columns in 2.0 mL collection tubes[11,12], membrane-anchored spin columns in 2.0 mL collection tubes[12,13], phenol/guanidine-based lysis reagent, wash buffer containing guanidine and ethanol (wash buffer 1), wash buffer containing ethanol (wash buffer 2), and RNase-free water.

4.2. Put a 10 mg kidney sample in the silicon homogenizer. Add 700 µL of the phenol/guanidine-based lysis reagent in the homogenizer.

4.3. Prepare the homogenizer and then slowly press/twist the homogenizer's pestle against the kidney sample to homogenize the sample. Repeat the pressing/twisting until the kidney sample is completely dissolved in the phenol/guanidine-based lysis reagent.

4.4. To further homogenize the sample, transfer the homogenized lysate (in a 2.0 mL collection tube) to the biopolymer spin column.

4.5. Spin the homogenized lysate at 14,000 x *g* for 3 min at room temperature (RT), and then transfer the precipitated lysate to an unused 1.5 mL microcentrifuge tube.

4.6. Combine the lysate in the tube with 140 µL of chloroform and then close the tube cap tightly. To mix the lysate and chloroform, invert the tube 15 times.

NOTE: The chloroform can be used without a hood.

4.7. Incubate each sample for 2–3 min at RT and then spin each sample at 12,000 x *g* for 15 min at 4°C.

4.8. Without disturbing the precipitate, transfer the supernatant (which is typically ~300 µL) to a new 1.5 mL microcentrifuge tube, and then add 1.5x its volume (typically ~450 µL) of 100% ethanol. Vortex the mixture for 5 s.

4.9. Load 700 µL of the sample onto a membrane-anchored spin column in a 2.0 mL collection tube. Close the column cap and spin the column at 15,000 x *g* for 15 s. Throw away the precipitated lysate in the collection tube.

4.10. Wash the sample thoroughly by adding 700 µL of wash buffer 1 to the membrane-anchored spin column in a 2.0 mL collection tube. Close the column cap, and spin the column at 15,000 x *g* for 15 s. Throw away the precipitated lysate in the collection tube.

4.11. Load 500 µL of wash buffer 2 onto the membrane-anchored spin column in a 2.0 mL collection tube to remove trace salts. Close the column cap, and spin the column at 15,000 x *g* for 15 s. Throw away the precipitated lysate in the collection tube.

NOTE: A membrane-anchored spin column can separate RNA and DNA.

4.12. Perform step 4.11 again.

4.13. Spin the membrane-anchored spin column in a 2.0 mL collection tube again at 15,000 x *g* for 1 min. Throw away the precipitated lysate in the collection tube.

4.14. Transfer the membrane-anchored spin column to a new 1.5 mL collection tube. Dissolve total RNA by adding 30 µL of RNase-free water to the column. Close the column cap, and wait 5 min at room temperature. Then, spin the column at 15,000 x *g* for 1 min.

4.15. Transfer the total amount of sample containing total RNA to a new microcentrifuge tube. Put each of the tubes on ice, and measure the concentration of total RNA by spectrophotometry.

4.16. Keep the tubes with samples at −80 °C for long-term storage before use.

5. Synthesis of cDNA with the reverse transcription of total RNA

NOTE: The MIQE (Minimum Information for the Publication of Quantitative Real-Time PCR Experiments) guidelines were issued to encourage better experimental practices and help obtain reliable and unequivocal results[19]. In this protocol, cDNA is synthesized from 1.0 µg of purified total RNA in a two-step procedure using reverse transcriptase, poly(A) polymerase, and oligo-dT primer.

5.1. Prepare the following: 1.5 mL microcentrifuge tubes, eight-well strip tubes with caps, the cap of each eight-strip tube, distilled water, ice, a reverse transcriptase kit (see the **Table of Materials**)[14,15] in the melted state, a thermal cycler, and a vortex mixer.

5.2. Start the thermal cycler.

5.3. Prepare a master mix solution: Add 2.0 µL of reverse transcriptase mix (included in the kit) and 2.0 µL of 10x nucleic acid mix into 4.0 µL of 5x hi-spec buffer (to obtain a total of 8.0 µL master mix per tube).

5.4. Put 8.0 µL of the master mix solution into each tube of an eight-well strip tube.

5.5. Adjust the total RNA density. To isolate 1.0 µg of total RNA from the kidney samples in 12 µL of RNase-free water, transfer the appropriate amount of total RNA into distilled water, using the density data measured as described in step 4.15.

NOTE: If any DNA contamination is present, the contaminated DNA will be co-amplified in the qRT-PCR.

5.6. Place a 12 µL aliquot of total RNA into each tube and close the tube's cap. Centrifuge the tube for 15 x.

5.7. Put the tube in the thermal cycler and incubate the sample for 60 min at 37 °C. Next, immediately incubate the sample for 5 min at 95 °C for the synthesis of cDNA.

5.8. When the incubation is complete, transfer the cDNA into a new 1.5 mL microcentrifuge tube and dilute the cDNA ten times (1:10) with distilled water. Vortex and centrifuge the tube for 5 s.

5.9. Temporarily store the diluted cDNA on ice and move the samples to −80 °C for long-term storage before use.

6. qRT-PCR of miRNA

NOTE: We used the intercalator method to perform the qRT-PCR of miRNA. Primers for RNA are U6 small nuclear 2 (RNU6-2), miRNA-3070-3p, miRNA-6401, miRNA-7218-5p, and miRNA-7219-5p were used.

6.1. Prepare the following: 1.5 mL microcentrifuge tubes, a vortex mixer, a 96-well reaction plate for the qRT-PCR, adhesive film for the 96-well reaction plate, an adhesive film applicator, a 96-well centrifuge rotor, miRNA-specific primers, a real-time PCR instrument, and a green dye-based PCR kit (see the **Table of Materials**)[14,15] containing 2x PCR master mix and 10x universal primer.

6.2. After mixing them in 1.5 mL microcentrifuge tubes , vortex the following items: 6.25 µL of distilled water, 1.25 µL of each 5 µM miRNA primer dissolved in nuclease-free water, 12.5 µL of 2× PCR master mix, and 2.5 µL of 10x universal primer.

6.3. Prepare the cDNA synthesized as described in step 5, and melt it. Vortex and centrifuge the cDNA for 5 s.

6.4. Put a 22.5 µL aliquot of the reagent (created as described in step 6.2) in each well of the 96-well plate.

6.5. Put a 2.5 µL aliquot of cDNA in each well of the plate.

6.6. Using the adhesive film applicator, secure the adhesive film tightly on the 96-well plate. Centrifuge the plate with the 96-well centrifuge rotor at 1,000 x *g* for 30 s to settle the reactions at the bottom of each well.

7. PCR cycling

7.1. Start the real-time PCR system, and place the plate created as described in step 6.6. in the real-time PCR system. Set the experiment properties next; identify the experiment name. Select the following: "96-well (0.2 mL)" as the system's experiment type, "Comparative CT (ΔΔCT)" as the quantitation method, "SYBR Green Reagents" as the reagents to detect the target sequence, and "standard" as the system's run.

7.2. Assign a name to the sample and the target miRNA, and then assign a name to the sample and target miRNA in each well. Samples should be assigned in duplicate in order to obtain appropriate data for confirmation of the results. Select a reference sample and an endogenous control, and select "none" for the dye to be used as the passive reference. Make sure to set the negative reverse transcriptase and non-template control for the miRNA expression in order to eliminate the cross-contamination of reagents.

7.3. Make sure the reaction volume setting is "20 µL" and the PCR cycling conditions are set at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 70 °C for 30 s.

7.4. After the qRT-PCR process is complete, use the system's software program to analyze the qRT-PCR data. Ensure that the threshold line that is automatically selected by the software program is appropriate for each well.

7.5. Check the threshold cycle (CT) value of the endogenous control and target miRNA analyzed in each sample. The CT value is determined by the intersection of the amplification curve and the threshold line. In the present study, we used RNU6-2 as an endogenous control for the target miRNA expression level, and we used the ΔΔCT method to determine the relative expression level of each target miRNA[20].

REPRESENTATIVE RESULTS:

A UUO mouse model was created by left ureteral ligation as described[21] in 8-week-old male mice weighing 20–25 g. Ureters were completely obstructed by double ligation with 4-0 silk sutures. An analgesic (meloxicam 5 mg/kg, subcutaneous injection) was administered before surgery and also daily on the 2 days post-surgery. At 8 days post-surgery, kidneys were collected, rinsed with PBS, dissected, and stored in liquid nitrogen for further analysis. Sham-operated mice served as controls. The double ligation is successful if the left kidney has hydronephrosis. Based on the miRNA qRT-PCR data obtained using this UUO model, the level of miRNA-3070-3p was significantly increased and the levels of miRNA-7218-5p and miRNA-7219-5p were considerably decreased in the kidneys of the UUO mice compared to the controls (**Figure 1**).

FIGURE AND TABLE LEGENDS:

Figure 1: Differentially expressed miRNAs in the kidneys of UUO mice. qRT-PCR analysis of miRNA-3070-3p, miRNA-6401, miRNA-7218-5p, and miRNA-7219-5p expression in sham mice (n=8) and UUO mice (n=8). Values are mean ± standard error (error bars). T-tests were used to investigate significant differences between groups. T-tests with a p-value <0.05 were

considered significant. miRNA: microRNA, n.s.: not significant, qRT-PCR: quantitative real-time reverse-transcription polymerase chain reaction, UUO: unilateral ureteral obstruction. *p<0.05. Click here to view a larger version of this figure.

DISCUSSION:

The above-described protocol with qRT-PCR successfully determined the expression levels of the targeted miRNAs. The assessment of extracted miRNAs is important when seeking to obtain meaningful qRT-PCR data, and in order to confirm the quality of the miRNAs before performing the qRT-PCR, the ratio of absorbance at 260 nm to that at 280 nm should be checked with a spectrophotometer. If a single PCR amplification of the expected length and melting temperature or a monomodal melting curve cannot be obtained by qRT-PCR, there may be DNA contamination or a primer dimer in each well of the reaction plate.

The expression levels of miRNAs can be evaluated by several methods other than qRT-PCR, including microarray, northern blotting, and ribonuclease protection assay methods. However, qRT-PCR is a simple, highly reproducible procedure that is both accurate and sensitive, and smaller sample volumes can be used for qRT-PCR compared to northern blotting and ribonuclease protection assays[22]. In addition, since microarrays enable the simultaneous measurement of the expression of tens of thousands of miRNAs, they can identify candidate miRNA markers. Microarray data have shown an overall high correlation with data obtained by qRT-PCR[23], but a consensus on the optimal methodology for comparing the microarray data obtained in disparate studies has not been reached[24].

The new protocol has the following limitations. The utility of this protocol has not been validated in other organs, such as liver and lung; and the protocol has not been tested in other laboratory animals (e.g., rats, dogs, and pigs). Several groups reported that this protocol (for the purification and detection of miRNAs by qRT-PCR) enabled the purification of high-quality RNA from tissues[12,14,15]. The method has high accuracy and sensitivity for detecting miRNA expression[12,14]. The present report demonstrates that this protocol can be used to successfully detect miRNA expression in mouse kidney. The protocol can thus be used to determine the miRNA expression profiles in the kidneys of mice with a wide range of disease states. Due to the protocol's simplicity, many samples can be processed simultaneously, and using the protocol can therefore contribute to the analyses of the expression of many miRNAs in various pathological conditions of the kidney.

There are certain aspects of the protocol to be aware of and keep in mind. First, the purified RNAs must be kept on ice to prevent degradation at room temperature. The kidney samples must be homogenized until the samples are completely melted in the lysis reagent. Since mouse kidneys contain substantial connective tissue that does not dissolve in lysis reagent, a column shredder is necessary for further homogenization. Second, the proper endogenous control miRNA (whose expression is stable among samples) must be verified throughout the qRT-PCR experimental setup because the invasion of various substances under this protocol could change the expression level of the endogenous control miRNA, possibly compromising the results.

In conclusion, we have presented the details of a qRT-PCR protocol for the detection, purification, and evaluation of microRNA expressions in mouse kidney with UUO.

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DISCLOSURES:

The authors declare that they have no conflicts of interest.

REFERENCES

1 Liu, B. et al. Identifying functional miRNA-mRNA regulatory modules with correspondence latent dirichlet allocation. *Bioinformatics.* **26** (24), 3105-3111 (2010).

2 Rottiers, V., Naar, A. M. MicroRNAs in metabolism and metabolic disorders. *Nature Review Molecular Cell Biology.* **13** (4), 239-250 (2012).

3 Roy, S. miRNA in Macrophage Development and Function. *Antioxidants and Redox Signaling.* **25** (15), 795-804 (2016).

4 Sun, Z. et al. Effect of exosomal miRNA on cancer biology and clinical applications. *Molecular Cancer.* **17** (1), 147 (2018).

5 Zhou, W. C., Zhang, Q. B., Qiao, L. Pathogenesis of liver cirrhosis. *World Journal of Gastroenterology.* **20** (23), 7312-7324 (2014).

6 Schuler, E., Parris, T. Z., Helou, K., Forssell-Aronsson, E. Distinct microRNA expression profiles in mouse renal cortical tissue after 177Lu-octreotate administration. *PLoS One.* **9** (11), e112645 (2014).

7 Blasco-Baque, V. et al. Associations between hepatic miRNA expression, liver triacylglycerols and gut microbiota during metabolic adaptation to high-fat diet in mice. *Diabetologia.* **60** (4), 690-700 (2017).

8 Cohen, A., Zinger, A., Tiberti, N., Grau, G. E. R., Combes, V. Differential plasma microvesicle and brain profiles of microRNA in experimental cerebral malaria. *Malaria Journal.* **17** (1), 192 (2018).

9 Gao, F. et al. Therapeutic role of miR-19a/19b in cardiac regeneration and protection from myocardial infarction. *Nature Communications.* **10** (1), 1802 (2019).

10 Xie, W. et al. miR-34b-5p inhibition attenuates lung inflammation and apoptosis in an LPS-induced acute lung injury mouse model by targeting progranulin. *Journal of Cellular Physiology.* **233** (9), 6615-6631 (2018).

11 Clark, R. M., Coffman, B., McGuire, P. G., Howdieshell, T. R. Myocutaneous revascularization following graded ischemia in lean and obese mice. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy.* **9** 325-336 (2016).

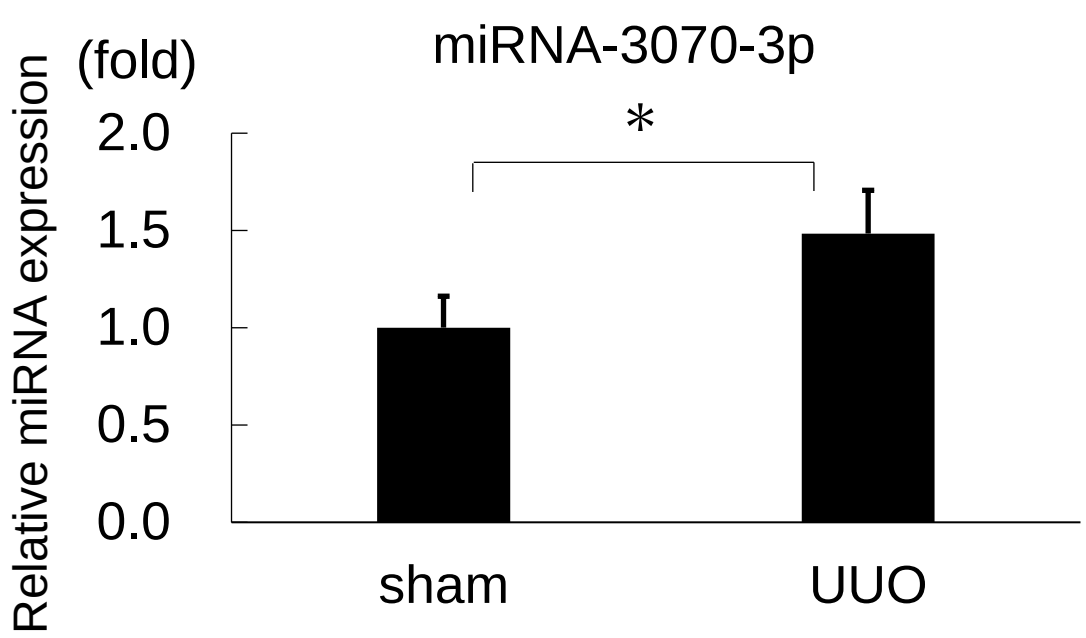
12 Morse, S. M., Shaw, G., Larner, S. F. Concurrent mRNA and protein extraction from the same experimental sample using a commercially available column-based RNA preparation kit. *Biotechniques.* **40** (1), 54, 56, 58 (2006).

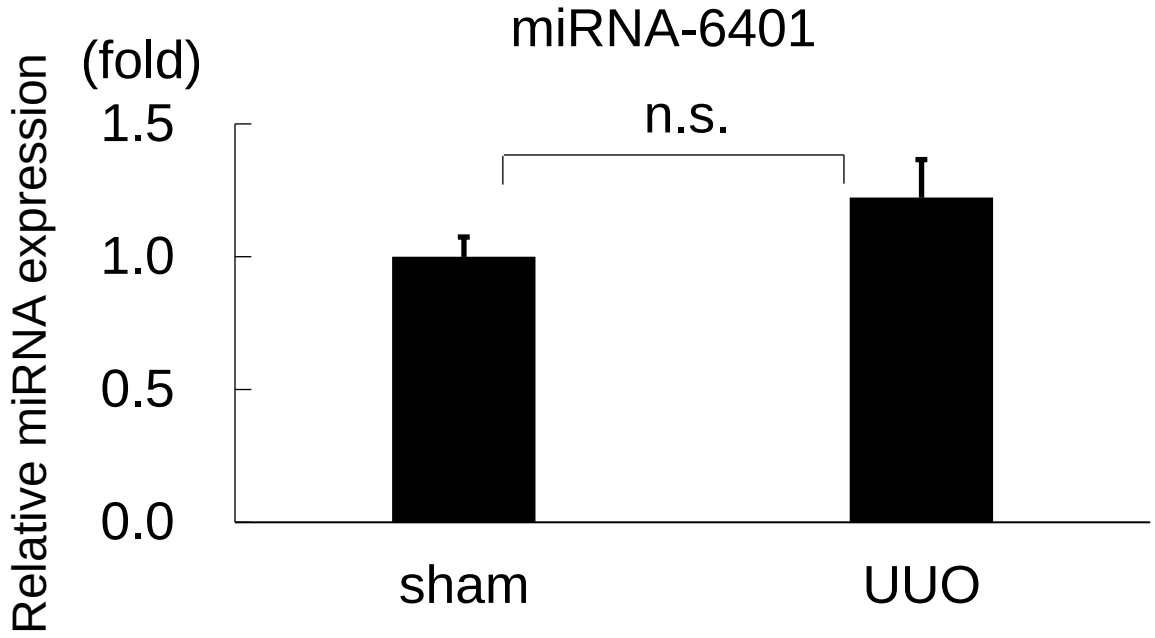
13 Sellin Jeffries, M. K., Kiss, A. J., Smith, A. W., Oris, J. T. A comparison of commercially-available automated and manual extraction kits for the isolation of total RNA from small tissue samples. *BMC Biotechnology.* **14** 94 (2014).

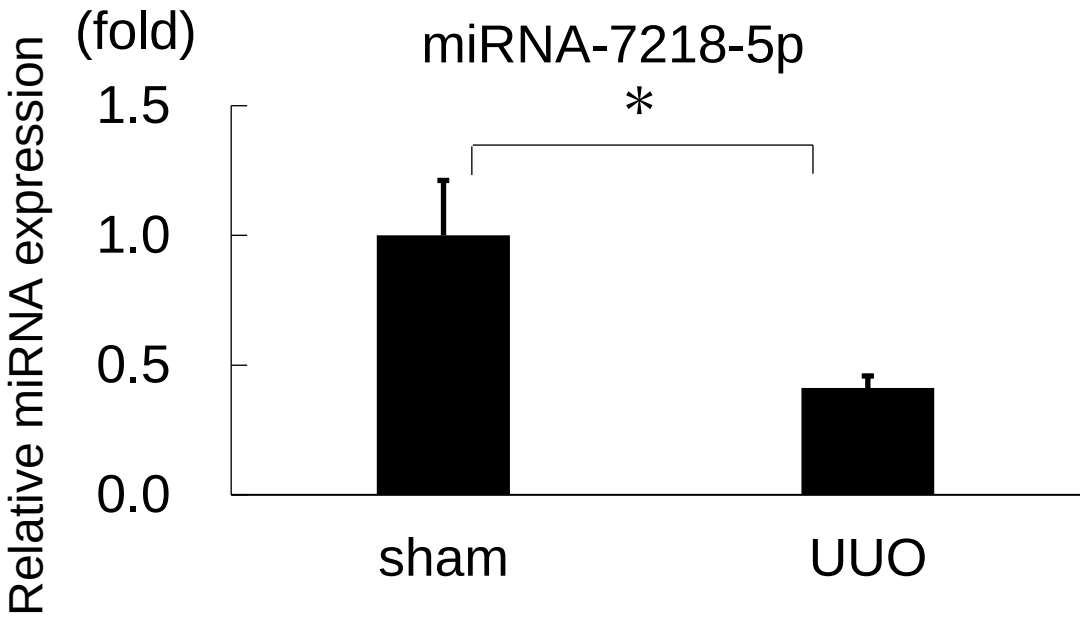
14 Mestdagh, P. et al. Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. *Nature Methods.* **11** (8), 809-815 (2014).
15 Kang, K. et al. A novel real-time PCR assay of microRNAs using S-Poly(T), a specific oligo(dT) reverse transcription primer with excellent sensitivity and specificity. *PLoS One.* **7** (11), e48536 (2012).
16 Lv, W. et al. Therapeutic potential of microRNAs for the treatment of renal fibrosis and CKD. *Physiological Genomics.* **50** (1), 20-34 (2018).
17 Liu, S. H. et al. C/EBP homologous protein (CHOP) deficiency ameliorates renal fibrosis in unilateral ureteral obstructive kidney disease. *Oncotarget.* **7** (16), 21900-21912 (2016).
18 Buchtler, S. et al. Cellular Origin and Functional Relevance of Collagen I Production in the Kidney. *Journal of the American Society Nephrology.* **29** (7), 1859-1873 (2018).
19 Bustin, S. A. et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry.* **55** (4), 611-622 (2009).
20 Rao, X., Huang, X., Zhou, Z., Lin, X. An improvement of the 2^(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostatics Bioinformatics and Biomathematics.* **3** (3), 71-85 (2013).
21 Chevalier, R. L., Forbes, M. S., Thornhill, B. A. Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy. *Kidney International.* **75** (11), 1145-1152 (2009).
22 Radonic, A. et al. Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications.* **313** (4), 856-862 (2004).
23 Zubakov, D. et al. MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. *International Journal of Legal Medicine.* **124** (3), 217-226 (2010).
24 Brazma, A. et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nature Genetics.* **29** (4), 365-371 (2001).

Figure

Fig. 1







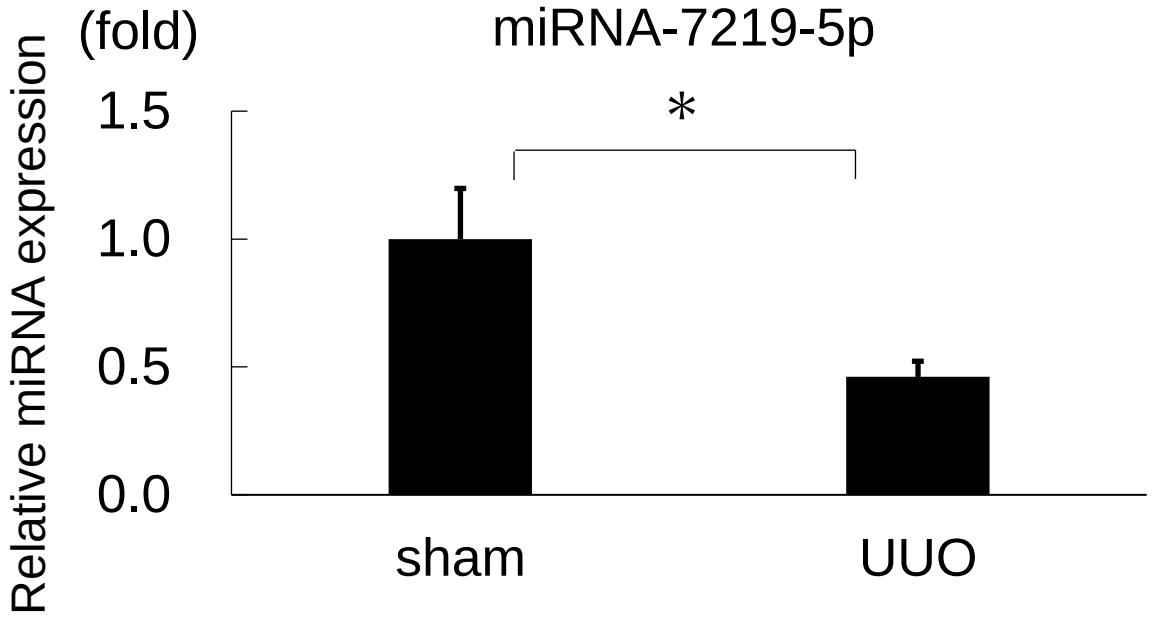


Table of Materials

Name of Material/ Equipment	Company	Catalog or Model No.
Buffer RPE	Qiagen	79216
Buffer RWT	Qiagen	1067933
C57BL/6 male mice	Tokyo Laboratory Animals Science	Not assigned
MicroAmp Optical 96-well reaction plate for qRT-PCR	Thermo Fisher Scientific	4316813
MicroAmp Optical Adhesive Film	Thermo Fisher Scientific	4311971
miRNA-3070-3p primer	Qiagen	MS00001701
miRNA-6401 primer	Qiagen	MS00065141
miRNA-7218-5p primer	Qiagen	MS00068067
miRNA-7219-5p primer	Qiagen	MS00068081
miRNeasy Mini kit	Qiagen	217004
miScript II RT kit	Qiagen	218161
miScript SYBR Green PCR kit	Qiagen	218073
QIA shredder	Qiagen	79654
QIAzol Lysis Reagent	Qiagen	79306
QuantStudio 12K Flex Flex Real-Time PCR system	Thermo Fisher Scientific	4472380
QuantStudio 12K Flex Software version 1.2.1.	Thermo Fisher Scientific	4472380
RNase-free water	Qiagen	129112
RNU6-2 primer	Qiagen	MS00033740
Takara biomasher standard	Takara Bio	9790B
4-0 silk	ASKUL	GA04SW
4-0 nylon	AS ONE	ER1004NA45-KF2,62 -9968-32

Comments/Description
Wash buffer 2
Wash buffer 1
96-well reaction plate
Adhesive film for 96-well reaction plate
5'-UUAAUGCUAAUUGUGAUAGGGGU-3'
5'-UUACACUCCAGUGGUGUCGGGU-3'
5'-UGCAGGGUUUAGUGUAGAGGG-3'
5'-UGUGUUAGAGCUCAGGGUUGAGA-3'
Membrane anchored spin column in a 2.0 mL collection tube
Reverse transcriptase kit
Green dye-based PCR kit
Biopolymer spin columns in a 2.0 mL collection tube
Phenol/guanidine-based lysis reagent
Real-time PCR instrument
Real-time PCR instrument software
Not disclosed
Silicon homogenizer

July 21, 2020

Topic Editors
Journal of Visualized Experiments

Revised ms. #61383R1: "Quantitative real-time PCR evaluation of microRNA expressions in mouse kidney with unilateral ureteral obstruction"

Dear Topic Editors,

We thank the Editor and Reviewers for providing thoughtful comments regarding our above-entitled manuscript. We have revised the text in accord with the Reviewers' suggestions, and we believe that these revisions have strengthened the study and manuscript. Our responses to the Editor and Reviewers' comments are provided below. Please address all correspondence to me, using the contact information given below.

We hope that our manuscript is now suitable for publication in *JoVE*.

Best regards,

Yoshiyuki Morishita, MD, PhD
Division of Nephrology
Department of Integrated Medicine
Saitama Medical Center
Jichi Medical University
1-847 Amanuma, Omiya, Saitama 330-8503, Japan
Tel.: +81-48-647-2111, Fax: +81-48-647-6831
Email: ymori@jichi.ac.jp

Revised ms. #61383R1: "Quantitative real-time PCR evaluation of microRNA expressions in mouse kidney with unilateral ureteral obstruction"

Response to an Editor's Comment

Editorial comments:

You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.

NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text on lines 37-39, 145-149, 154-159, 166-174, 176-182, 187-192, 220-222 to avoid this overlap.

Response: Thank you for all of the helpful comments. New text in the manuscript is presented in red font.

We re-wrote the text at the above-cited lines.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:

1) 3.4: unclear what is meant by "reflux it".

2) 4.6: Add a note to caution for chloroform use. Is this done under a hood?

3) 4.11: mention spin column specs.

Response: The wording "reflux it" meant to wash out blood from vessels.
Chloroform can be used without a hood. We added this caution in the protocol.
We also added spin column specs: "(Note: A membrane-anchored spin column can separate RNA and DNA)."

• **Protocol Numbering:**

1) All steps should be lined up at the left margin with no indentations.

2) Add a one-line space between each protocol step.

Response: We revised the text in accord with these instructions.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

Response: We put the highlighted steps within 2.5 pages.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and

in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: We have done so.

• **References:** Please spell out journal names.

Response: We have done so.

• **Table of Materials:**

1) Sort the list alphabetically.

Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Response: We have done so.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response to a Reviewer's Comment

Reviewer 1's Comment:

Major Concerns:

For both the sham and UUO surgery the protocol says "Euthanize a mouse with an overdose of isoflurane, then apply depilatory cream to the mouse abdomen". I assume this is not what the

authors meant as this would be performing surgery on a dead mouse. This part of the protocol should precisely detail the surgery set up in mice that are anaesthetised by isoflurane. The authors make no mention of the intestines, standard operating for this model is to carefully pull the intestines to the side and place in moistened swab to allow a clear field of view of the ureter.

Response: Thank you for all of the helpful comments. New text in the manuscript is presented in red font.
We have revised the protocol terms and added step 1.5. We re-wrote step 2.5.

I am also concerned that there is no mention or pre-operative or peri-operative analgesic administration. Furthermore in the standardised good care of animals these animals should have a 2nd analgesic dose administered 24hrs post surgery.

Response: We added the concentration of isoflurane in step 1.2. In addition, we described the subcutaneous injection of analgesics.

The suture choice is very thick for mice, what are the advantages to using 4-0 over 5 or 6-0.

Response: Our examination of the past references revealed more references that used 4-0 silk than 5-0 or 6-0 silk in performing UUO surgery, so we used 4-0 silk.

For the qRT-PCT the authors state "NOTE: qRT-PCR can be performed using total RNA as a template without reverse transcription for quality control of purified total RNA. If there is any DNA contamination, unexpected amplification products will be observed upon qRT-PCR". This is an essential control for determination of miRNA expression. There should always be a negative RT included in all runs to detect and amplification of genomic DNA or contamination. This is not an optional extra and should be included as per MIQE.
A further required control is the presence of non-template controls (NTC) for miRNA expression. This is not clear in the current methodology.

Response: We added negative RT and non-template controls to protocol.

In the discussion mention is made of the importance of melting curves for miRNA expression. Checking the melting curve should be added to the methodology as its very important that the primers are only detecting a single amplicon. The issue of primer dimer should also be discussed.

Response: As you suggested, we added the following to the Discussion: " If a single PCR amplification of the expected length and melting temperature or a monomodal melting curve cannot be obtained by qRT-PCR, there may be DNA contamination or a primer dimer in each well of the reaction plate."

The amount of input tissue is very high and may exceed the binding capacity of the silica membrane. The authors should have tested differing amounts and reflected on the optimum amount required.

Response: We agree, a sufficient amount of miRNA can be extracted with a 10-mg kidney sample. We re-wrote this part of the text.

Minor Concerns:

The terminology of making Sham and UUO mice, this does not reflect the methodology, this should be performing the sham and UUO surgery. For the sham surgery the inclusion of the manipulation of the ureter is important to add.

Response: We re-wrote this description.

The sex of the mice affects how the surgery is performed slightly, no mention is made of sex or the difference this has.

Response: In the prior studies, UUO surgery was often done in males, so we performed UUO surgery in males.

For homogenisation of tissue in phenol/guanidine based solutions, this needs to be performed at room temperature, this is important for efficient lysis.

Response: As you pointed out, homogenization needs to be performed at room temperature. We have now noted this.

Reviewer 2's comment:

Major Concerns:

"RNA is reverse-transcribed using reverse transcriptase, poly(A) polymerase, and oligo-dT primer". Is this a one step or two step procedures? My understanding is that for these miRNAs without polyA tails, poly A should be added to the 3' terminus of miRNA before reverse transcriptase starts to work, therefore one step reaction might not provide high efficacy.

Response: Thank you for all of the helpful comments. New text in the manuscript is presented in red font.

We perform two-step procedures. We have revised step 5.

Minor Concerns:

In introduction, second paragraph, "Next, miRNA-containing total RNA was purified from .....", It should be "Next, total RNA containing miRNA was purified from ....."

Response: We have revised the text as suggested.

Response: We are very grateful to the Reviewer for this important advice. As suggested, we have revised our manuscript. We believe that these changes have greatly improved the quality of the manuscript.