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TITLE:

A Quantitative Detection Method for MicroRNAs in the Kidney of an Ischemic Kidney Injury Mouse Model

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KEYWORDS:

MicroRNA, mouse, acute kidney injury, ischemic reperfusion model, purification, detection, qRT-PCR

SUMMARY:

This article presents a protocol for detecting microRNA expression in the kidneys of an acute kidney injury mouse model using quantitative real-time reverse-transcription polymerase chain reaction. This protocol emphasizes an ischemic kidney injury mouse model and the careful extraction of microRNA samples.

ABSTRACT:

MicroRNAs (miRNAs) are involved in various disease states and are effective biomarkers for the early diagnosis of diseases and treatment in mice. However, standard protocols for the purification of miRNAs and detection of their expression in the kidneys of acute kidney injury (AKI) mice have not been well established. This study developed an effective and simple protocol to purify and quantify miRNAs in the kidneys of an AKI mouse model induced by renal ischemia-reperfusion using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). This protocol comprises five steps: 1) induction of AKI by renal ischemia-reperfusion, 2) harvesting of kidneys, 3) purification of total RNA, including miRNAs, from kidneys, 4) cDNA synthesis by reverse transcription of miRNA, and 5) qRT-PCR to detect miRNA expression. Using this protocol, the renal ischemia-reperfusion injury model can be generated with mild to severe

forms of AKI. Additionally, if the procedure is followed properly, a consistent AKI model with minimal individual differences can be obtained. This qRT-PCR assay shows a very wide dynamic range and enables the discrimination of mature miRNAs, which can be accurately quantified with high specificity. This protocol can be used to study the miRNA expression profile in AKI kidneys.

INTRODUCTION:

Ischemia-reperfusion injury (IRI) of the kidney represents one of the major risk factors for Acute kidney injury (AKI) development[1]. AKI plays a significant role in patient prognosis, but specific therapies and early diagnostic biomarkers have not been established.

MicroRNAs (miRNAs) are short, non-coding RNAs with approximately 18–25 bases. miRNAs are stable in body fluids, and their sequences are highly conserved among animals[2]. MiRNAs regulate the expression of multiple proteins through thousands of targets, thereby influencing diverse signaling pathways[2-8]. In recent years, it has been reported that miRNAs are involved in various disease states and are effective biomarkers for the early diagnosis of several diseases.

The overall goal of this protocol is to successfully purify and detect miRNAs in the kidneys of an AKI mouse model induced by IRI. This IRI model is a widely used model of AKI and renal fibrosis. The advantages of the IRI model include being able to visually confirm whether ischemia-reperfusion has been achieved and determine the exact time of AKI onset. However, a clamp duration that is long enough to cause broad tubular damage is associated with a high mortality rate, whereas a short clamp duration does not cause tubular damage, which results in a large variation in tubular damage progression in this experimental model. Compared with the bilateral clamping model, the right nephrectomy tissue harvested in the unilateral renal IRI model acts as a control and ensures renal failure.

The kidney sample is mashed using a glass homogenizer, wherein, proteins and nucleic acids are separated, and DNA and RNA are separated[9]. Total RNA, including miRNA, is purified from the kidney sample using a silica-membrane-based spin column[9]. Subsequently, cDNA synthesis (reverse transcription) is performed from total RNA using poly(A) polymerase and oligo-dT primers[10]. Finally, the miRNA expression is determined by qRT-PCR using an intercalating dye[10]. qRT-PCR can more accurately quantify gene expression compared with PCR of amplification volumes at endpoints. qRT-PCR measures high concentrations and is characterized by a wide dynamic range, which allows accurate quantification that depends on the number of cycles. Previous studies reported that simple processes could be used to effectively purify and detect miRNAs in tissues[8-10]. The methods described in this protocol for cDNA synthesis (reverse transcription) and the detection of miRNA expression by qRT-PCR using an intercalating dye have been reported to show high accuracy and sensitivity[10].

Additionally, this protocol is simple and achieves consistent results between laboratories. Therefore, this protocol is useful in studies that require highly accurate and sensitive miRNA detection in mouse AKI kidneys.

PROTOCOL:

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

1. IRI model

NOTE: Carefully monitor for hypothermia, intestinal moisturization, and depth of anesthesia throughout the procedure.

1.1. Prepare the following items: a 50 mL centrifuge tube containing cotton drenched in isoflurane, a heated surgical pad, a Petri dish with phosphate-buffered saline (PBS), surgical scissors, and forceps. Maintain the PBS at 37 °C.

NOTE: This IRI model was generated using male C57BL/6 mice (9 weeks; 20–25 g) housed in a room with controlled temperature, humidity, and a 12 h light-dark cycle.

1.2. Anesthetize the animal using isoflurane: induction concentration 3%–5%, maintenance concentration 2%–3%, and concentration during clamping 1%–1.5%. Confirm the depth of anesthesia by the loss of reflexes. Inhalation anesthesia has the advantage that the time can be adjusted. The procedure might take a significant amount of time because of the exfoliation of the tissue surrounding the kidney. However, the disadvantage of inhalation anesthesia is that the body temperature tends to fall. Throughout the procedure, avoid unnecessary anesthesia.

1.3. Mount the anesthetized mouse on a heated surgical pad on its back, remove the hair surrounding the incision area, and spray the abdominal skin of the mouse with 70% ethanol.

1.4. Make a midline incision in the abdominal skin, muscle, and peritoneal membrane using scissors and forceps.

1.5. Using a cotton swab moistened with PBS, gently push the bowel toward the left side of the extra-abdominal cavity, exposing the right kidney and ureter. The long procedure time is likely to cause postoperative intestinal injury. Thus, it is better to operate without removing the intestines from the abdominal cavity when a visual field can be obtained. Avoid drying out the intestines. Dry swabs or gauze can cause intestinal damage.

1.6. Lift the right ureter with angled forceps. Ligate the right ureter twice using 4/0 silk-braided sutures. Cut between the sutures, leaving the suture that is closer to the kidney longer.

1.7. Carefully hold the remaining suture without pulling too much, and bluntly dissect the connective tissue and fat along the kidney. Performing surgery from behind reduces the risk of liver damage.

1.7.1. Identify a blood vessel, other than the renal arteries and veins, that supplies blood to the right kidney, and then ligate or clip it. When the right kidney is sufficiently detached, ligate the right renal artery and vein using a 4/0 silk-braided suture. Instead of suturing, use a hemostatic clip.

1.7.2. Using a cotton swab moistened with PBS, gently push the intestine towards the right side of the extra-abdominal cavity to expose the left kidney, and then cover the intestine with a moistened drape to prevent it from drying out. Dissect the connective tissue and fat around the left kidney.

1.8. Identify a blood vessel, other than the renal arteries and veins, that supplies blood to the left kidney, and then ligate or clip it. Clamp the renal artery and renal vein to induce left kidney ischemia. Position the hemostatic clip approximately 1 cm from the kidney to avoid damage to the renal parenchyma. Successful ischemia can be visually confirmed by a gradual uniform darkening of the kidney.

1.9. Return the intestines to the abdominal cavity, taking care to avoid intestinal twisting; temporarily close the abdominal skin and drape to maintain a moist environment. Reduce the concentration of inhalation anesthesia as much as possible and try to keep the animal warm. The clamping time is 25–45 min. A longer clamping time induces more severe renal damage but also increases long-term mortality. Adjust by analyzing the pathology results.

1.10. Remove the clamp after the period of ischemia has concluded. Ensure that blood flow reperfusion and kidney color improve. Confirm that the bowel is not twisted before closing the abdominal skin. Instill PBS intraperitoneally as a volume supplement before the abdomen is closed in two layers. Close the abdominal skin using 4-0 nylon sutures. For longer renal removal periods, the possibility of intestinal adhesions may be reduced by separately closing the abdominal wall and peritoneum.

1.11. To minimize the risk of post-operative infection, apply an antiseptic (e.g., iodine/alcohol solution) to the surgical area.

2. Kidney sample collection

NOTE: This video has a 45 min clamping time, collecting the kidneys 24 h later.

2.1. Anesthetize the animal with 5% isoflurane and confirm the depth of anesthesia by the loss of reflexes.

2.2. Make a midline incision in the abdominal skin, muscle, and peritoneal membrane using scissors and forceps. Collect the blood from the inferior vena cava punctured by a 27 G needle.

2.3. Make a midline incision in the chest wall and insert a 23 G needle into the left ventricle. Confirm backflow. Make an incision in the liver and inject 20 mL of PBS. As the blood drains, the liver changes color from red to pink, confirming that sufficient blood has been drained.

2.4. Remove the left kidney using forceps and scissors and wash it with PBS in a Petri dish. Remove the renal fascia using forceps, being careful not to dry out the kidney.

2.5. Cut the kidney in half and use one half for histopathology and the other half for the next step. Avoid the renal pelvis, and harvest 30 mg of the kidney. Store this at −80 °C before use.

3. Purifying miRNAs from kidney samples

NOTE: Here, kidney samples weighing 30 mg are homogenized using a glass homogenizer and a biopolymer-shredding system in a microcentrifuge spin column. Subsequently, miRNA from the kidney sample is isolated using a silica-membrane-based spin column.

3.1. Prepare the following items: 1.5 or 2.0 mL microcentrifuge tubes, 100% ethanol, chloroform, a glass homogenizer, ice, a biopolymer-shredding system in a microcentrifuge spin column[9], silica-membrane-based spin columns[9], phenol/guanidine-based lysis reagent, wash buffer containing guanidine and ethanol (wash buffer 1), and wash buffer containing ethanol (wash buffer 2).

3.2. Place a 30 mg kidney sample into a glass homogenizer and add 700 µL of phenol/guanidine-based lysis reagent. Perform this step on ice because the tissue is denatured by heat.

3.3. Homogenize the kidney sample by slowly pressing the pestle onto the sample by twisting it. Repeat this process several dozen times on ice until the kidney sample has completely dissolved into the phenol/guanidine-based lysis reagent. When miRNA is not obtained in subsequent measurements, this is likely because of insufficient dissolving.

3.4. For further homogenization, transfer the homogenized lysate to the biopolymer-shredding system in a microcentrifuge spin column placed in a 2.0 mL collection tube. Centrifuge this at 14,000 × *g* for 3 min at room temperature.

3.5. Transfer the homogenized lysate to a new microcentrifuge tube.

3.6. Add 140 µL of chloroform to the homogenized lysate and cap the tube securely. Mix the tube by inversion for 15 s.

3.7. Incubate the samples for 2–3 min at room temperature. Then, centrifuge them at 12,000 × *g* for 15 min at 4 °C.

3.8. Transfer the supernatant (normally 300 µL) to a new microcentrifuge tube without disturbing the precipitate and add 1.5 volumes (normally 450 µL) of 100% ethanol. Mix the sample by vortexing for 5 s.

3.9. Pipette up to 700 µL of the sample into a silica-membrane-based spin column placed in a 2.0 mL collection tube. Close the column cap, and centrifuge at 15,000 × *g* for 15 s. Following centrifugation, discard the flow-through in the collection tube.

3.10. Add 700 µL of wash buffer 1 to the silica-membrane-based spin column to thoroughly wash the sample. Close the cap of the column, and centrifuge it at 15,000 × *g* for 15 s. Following centrifugation, discard the flow-through in the collection tube.

3.11. Add 500 µL of wash buffer 2 to the silica-membrane-based spin column to remove any traces of salt. Close the cap of the column, and centrifuge at 15,000 × *g* for 15 s. Following centrifugation, discard the flow-through in the collection tube.

3.11.1. Repeat step 3.11.

3.12. Centrifuge the silica-membrane-based spin column again at 15,000 × *g* for 1 min. Following centrifugation, discard the flow-through in the collection tube.

3.13. Place the silica-membrane-based spin column in a new 1.5 mL collection tube. Transfer 25 µL of RNase-free water into the column, and close the column cap. Leave the sample at room temperature for 5 min, and then centrifuge it at 15,000 × *g* for 1 min.

3.14. Transfer the 25 µL eluate containing miRNAs to a new microcentrifuge tube. Because miRNA is degraded by repeated dissolution, it is recommended to dispense the sample into 2 or 3 tubes. Store these at −80 °C before use.

4. Reverse transcription of miRNA

NOTE: Here, 1.0 µg of isolated RNA is reverse-transcribed using reverse transcriptase, poly(A) polymerase, and oligo-dT primers.

4.1. Prepare the following items: 1.5 mL microcentrifuge tubes; 8-well strip tubes; RNase-free water; ice; a 10x Nucleic Acid Mix containing deoxynucleotides, ribonucleotide triphosphates, and oligo-dT primers; a poly(A) polymerase and reverse transcriptase mix; and activating buffer, including Mg^{2+}, deoxyribonucleotides, oligo-dT primers, and random primers [10].

4.2. Prepare a master mix solution containing 2.0 µL of 10x Nucleic Acid Mix, 2.0 µL of poly(A) polymerase and reverse transcriptase mix, and 4.0 µL of 5x activating buffer, including Mg^{2+}, deoxyribonucleotides, oligo-dT primers, and random primers, for a total of 8.0 µL per tube.

4.3. Dispense 8.0 µL of the master mix solution into each tube.

4.4. Add 1.0 µg of isolated miRNAs purified from the kidney sample to each tube.

4.5. Add RNase-free water to each tube to a total of 20 µL. Mix thoroughly by pipetting, and centrifuge at 1,500 x *g* for 15 s.

4.6. Incubate the samples for 60 min at 37 °C, followed immediately by incubation for 5 min at 95 °C. This step can be performed in a thermal cycler.

4.7. Transfer the cDNA to a new microcentrifuge tube and dilute 10 times (1:10) with RNase-free water.

4.8. Store the diluted cDNA short term on ice and long term at −80 °C before use.

5. qRT-PCR of miRNA

NOTE: qRT-PCR of miRNA is performed using an intercalating dye. Here, primers for U6 small nuclear 2 (RNA-2), miRNA-17-5p, miRNA-18a-5p, miRNA-21a-5p, miRNA-132-3p, miRNA-212-3p, miRNA-223-3p, and miRNA-574-5p were used.

5.1. Prepare the following items: 1.5 mL microcentrifuge tubes, 96-well reaction plates for qRT-PCR, adhesive film for the 96-well reaction plate, miRNA-specific primers, PCR Master Mix comprising Taq DNA polymerase, PCR buffer, deoxynucleotides, and universal primers[10], and a real-time PCR Instrument.

5.2. Prepare a master mix containing 12.5 µL of 2x PCR Master Mix, 2.5 µL of each miRNA primer (5 µM) dissolved in nuclease-free water, and 1.25 µL of 10x universal primers for each well.

5.3. Dispense 22.5 µL of the master mix into each well of the 96-well plate.

5.4. Add 2.5 µL of template cDNA to each well.

5.5. Seal the plate with the adhesive film for the 96-well reaction plate. Then, centrifuge the plate at 1,000 × *g* for 30 s.

5.6. Using the real-time PCR instrument and software, run the PCR cycling program as follows.

5.6.1. Place the plate in the real-time PCR instrument. Assign a name to the sample and target miRNA in each well using the Real-Time PCR software.

5.6.2. Input the following PCR cycling conditions in the real-time PCR software according to the instructions: preincubation 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.

5.7. Analyze the qRT-PCR data using the software of the real-time PCR instrument. Check for non-specific reactions. Analyze the Dissociation curve or the Melt curve to confirm that amplification other than the intended one has not occurred.

5.7.1. Normalize the expression level of target miRNAs to RNU-6 as an endogenous control. Calculate the relative expression level of target miRNAs using the $2^{-\Delta\Delta CT}$ method[11].

REPRESENTATIVE RESULTS:

Here, the miRNA expression profile in AKI mice was investigated. The miRNA expression profile has been investigated in various organs and tissues in mice. MiRNAs are important post-transcriptional regulators and are now being extensively studied in the characterization of a variety of diseases, including AKI. MiRNAs have the potential to help elucidate pathological conditions and be applied to the treatment of AKI[12]. The major causes of AKI are IRI, nephrotoxic insult, and sepsis. IRI of the kidney represents one of the major risk factors for the development of AKI. Numerous miRNAs have been reported to be involved in AKI[13].

This study investigated miRNAs that are commonly altered in sepsis and IRI models using the protocol described here. Seventeen miRNAs shown to be upregulated in the kidneys of AKI mice by microarray analyses were selected, and seven of these miRNAs reported to be expressed in humans were investigated. The levels of six miRNAs (miRNA-17-5p, miRNA-18a-5p, miRNA-21a-5p, miRNA-132-3p, miRNA-212-3p, and miRNA-223-3p) were found to be significantly increased in the kidneys of IRI mice compared with those in mock mice using qRT-PCR following the protocol presented in this manuscript (**Figure 1**).

FIGURE AND TABLE LEGENDS:

Figure 1: MiRNA expression in the kidney of AKI mice determined by qRT-PCR. MiRNAs in the kidneys of mice 24 h post-IRI. qRT-PCR determination of the expression of seven miRNAs (miRNA-17-5p, miRNA-18a-5p, miRNA-21a-5p, miRNA-132-3p, miRNA-212-3p, miRNA-223-3p, and miRNA-574-5p) in mock mice (n = 5) and IRI mice (n = 5). Six miRNAs (miRNA-17-5p[14], miRNA-18a-5p[15], miRNA-21a-5p[16], miRNA-132-3p[17], miRNA-212-3p[18], and miRNA-223-3p[19]) were shown to be upregulated by qRT-PCR. qRT-PCR analysis showed that miRNA-574-5p expression was unchanged. Values are the mean ± standard error (error bars). Analysis of variance and Student's t-tests were used to compare the differences among groups. Differences with a p-value < 0.05 were considered significant. Abbreviations: qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; miRNAs, microRNAs; NS, not significant; *, $p < 0.05$.

Figure 2: Serum blood urea nitrogen (BUN) and creatinine (CRE) levels 24 h post-IRI in mice (n = 5) and mock mice (n = 5).

Figure 3: Hematoxylin and Eosin (H&E) staining of tissues after 24 h in an IRI model with a clamp time of 45 min. Male C57/B6 mice aged 9 weeks underwent a right nephrectomy, and the left renal pedicle was clamped for 45 min. Mice were sacrificed at 24 h following renal IRI.

Representative photomicrographs (Magnification: 400×) of H&E-stained control and injured kidney sections obtained using an optical microscope. IRI induced acute tubular necrosis (black arrow).

DISCUSSION:

Using the protocol presented in this manuscript, miRNAs from the kidney of IRI mice were successfully purified and detected using qRT-PCR. Critical points of the IRI-inducing procedure in the protocol include careful monitoring of body temperature and anesthesia concentrations, which are known to affect AKI[20]. The strength of this protocol is that it allows visual confirmation of whether ischemia-reperfusion has been achieved. However, there are some limitations to this IRI model. Prolonging the clamp time to induce severe ischemia increases mortality. Additionally, when renal ischemia is not achieved using the left renal artery clamp, it is because blood vessels to the kidney other than the renal artery and vein are not ligated/clipped. There are alternative methods to induce AKI, including drug injection (folic acid, aristolochic acid, cisplatin, gentamycin, glycerol, L-mimosine, $CoCL_2$, kanamycin, and furosemide). In contrast to drug-induced AKI models, the time at which AKI is induced in the present model was accurately determined, with minimal individual differences in the AKI stage. In contrast to models using bilateral renal ischemia, IRI can be induced reliably using the protocol described here. In the present model, it is easy to administer drugs before and after IRI, including the application of miRNAs or related compounds that regulate genes or proteins, and the removed right kidney tissue can be used as a control.

There are three critical points regarding miRNA purification in this protocol. First, it is necessary to extract total RNA on ice because miRNA is pyrolyzed. It is also important to ensure that the kidney sample is properly homogenized and completely dissolved in the lysis reagent, or there will be a difference in the amount of miRNA collected. Second, confirming the stability of the expression levels of endogenous control, miRNAs is essential to accurately assess the level of miRNA expression required. It may be necessary to change the endogenous control depending on the condition, so make sure to determine whether there is a difference in the endogenous control between the control and IRI groups. RNU-6 was used here. When the endogenous controls are significantly different between the compared groups, interpretation of the results will be complicated. In that case, ensure that there are no differences in the conditions of the laboratory animals, animal feed, temperatures between samples, and extraction times. Third, the reliability of qRT-PCR data analysis depends on the quality of the purified miRNAs. If the amount of RNA contained in a sample is different, then the final results will be significantly different. When preparing cDNA, adjust the samples to ensure that the amount of RNA set between samples is the same. It is also necessary to carefully avoid technical errors caused by pipetting. Additionally, if significant miRNA amplification using qRT-PCR is not achieved, then consider increasing the concentration of template cDNA, concentration of each miRNA-specific primer, and number of qRT-PCR cycles.

This qRT-PCR protocol is an intercalator assay that detects any amplified double-stranded DNA, including non-specific reaction products. Although it has a high detection ability, and there is no need to prepare a special fluorescent-labeled probe for each target, it has the disadvantage that the intercalating dye itself may act as an inhibitor or bind to non-specific DNA sequences

and give rise to false positives. This is because all double-stranded DNA present in the reaction solution is detected, so non-specific amplification and target amplification are indistinguishable and detected as the same amplification. Therefore, it is necessary to confirm that there are no non-specific reactions after PCR.

First, analyze the Dissociation curve or the Melt curve, which are incorporated as a program in the real-time PCR device. The temperature at which double-stranded DNA dissociates into single strands is the melting temperature (Tm) value. The Tm value depends on sequence characteristics, such as the length of the amplification product and GC content. Therefore, if there is another amplification product, the Tm value will be different, and the change in the fluorescence value will be observed twice. If there is one peak Tm value, there is one amplification product, and if there are multiple peaks, there are multiple amplification products. If no amplification is achieved, then modify the conditions.

One parameter that can be changed is the primer concentration. Generally, a higher primer concentration is more likely to cause non-specific amplification. Therefore, lowering the primer concentration may improve non-specific amplification. Generally, the higher the primer concentration, the higher is the PCR efficiency. Therefore, it is recommended to first try using a high concentration, and then lower the concentration if the assay does not work.

Second, non-specific amplification tends to improve with increasing annealing/extension temperatures. Increase the annealing/extension temperature by 1 °C to determine whether the non-specific amplification disappears.

Third, the number of PCR cycles can be increased or decreased as needed. If only trace amounts of the miRNAs are detected, qRT-PCR may be difficult to use. When gene amplification is observed after 30 cycles, it is challenging to determine whether this is due to non-specific amplification, contamination, or whether a very small amount of specific nucleic acid was detected. The Melt curve is used as a reference, but the possibility of contamination cannot be excluded. In this case, consider using digital-PCR.

There are several alternative methods for determining the level of miRNA expression, such as northern blotting, ribonuclease protection, in situ hybridization, and microarrays, but qRT-PCR is faster and easier to perform than other RNA quantification methods. Furthermore, qPCR detection methods are more sensitive and specific than other assays. Microarrays can simultaneously measure the expression of multiple miRNAs, and the results are strongly correlated with the data obtained by qRT-PCR[21]. However, no methodology has been established to validate the comparison of microarray data between research groups[22].

The present protocol has the following limitations. Prolonging the clamp time to induce severe ischemia increases mortality. It is unsuitable for evaluating severe AKI models. Therefore, set the clamp time for each pathological tissue.

In conclusion, this article describes a protocol for the purification and detection of miRNA

expression in IRI mouse kidneys using qRT-PCR.

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DISCLOSURES:

The authors declare that they have no conflicts of interest.

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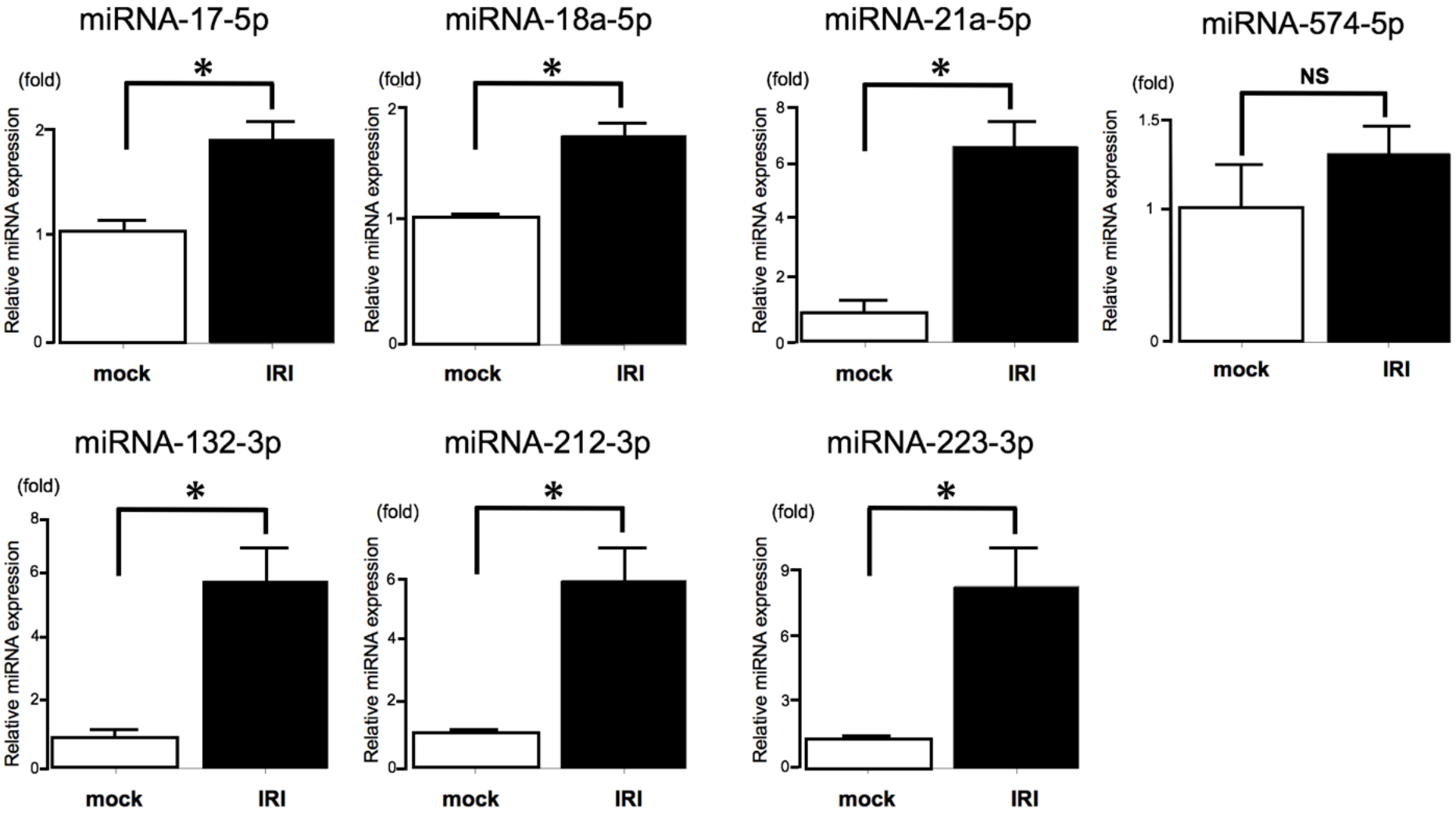
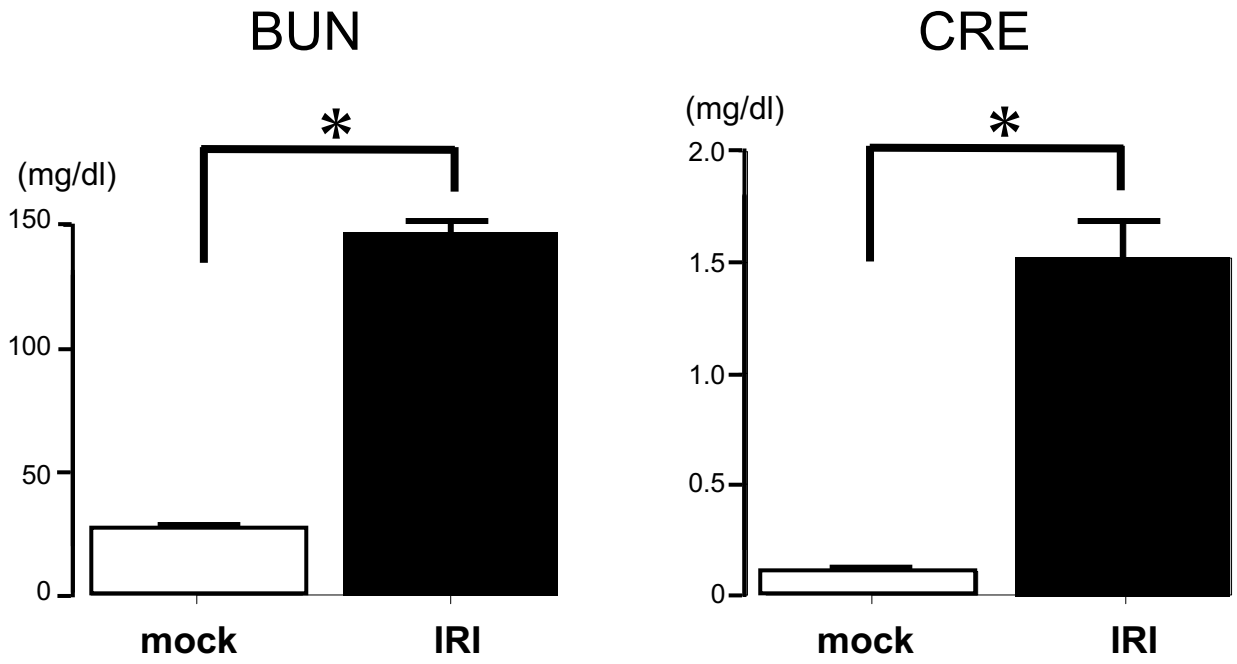
Figure



Figure



Figure

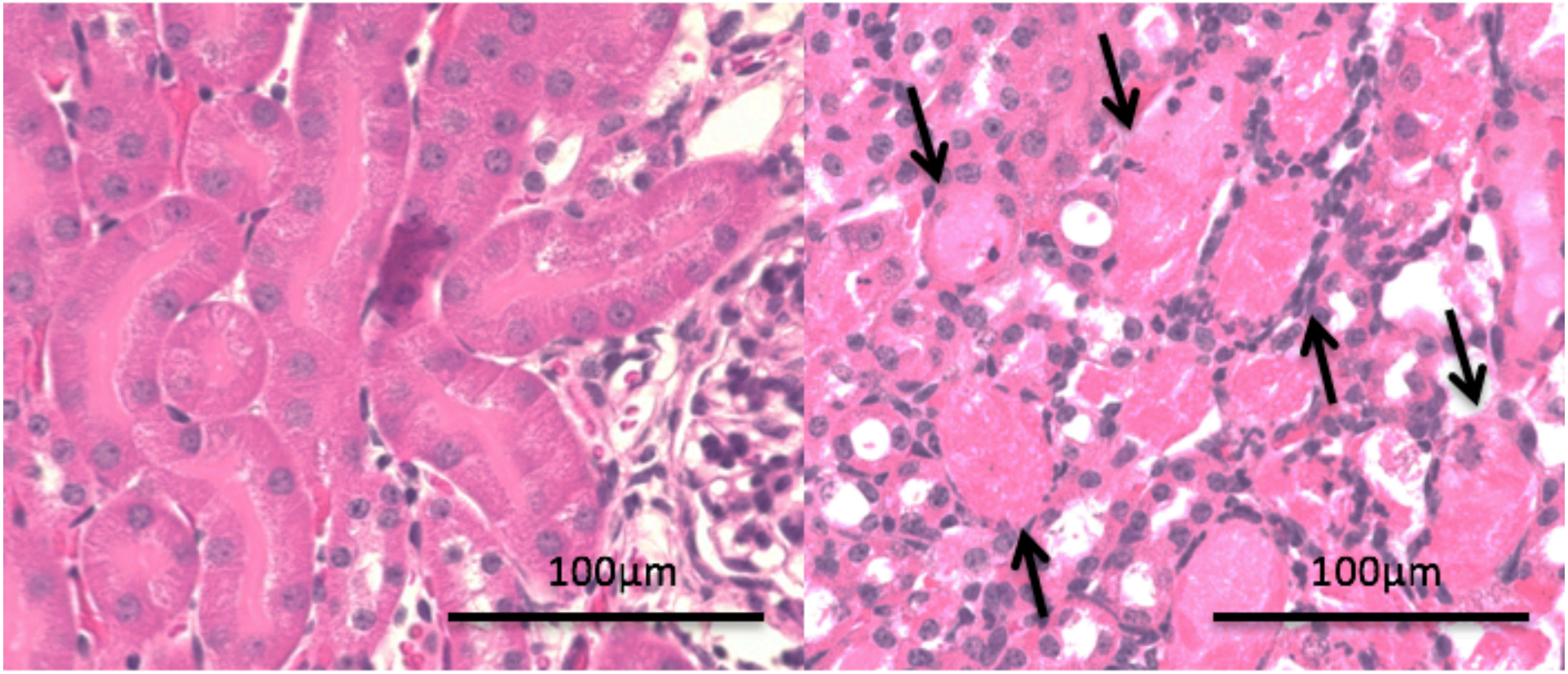


Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Bent Tip Tapered Tweezers without Hook	Natsume Seisakusho	MA-47	
Buffer RLT	Qiagen	79216	wash buffer 1
Buffer RWT	Qiagen	1067933	wash buffer 2
C57B6 mice	SLC	not assign	
forcep with Teeth	Natsume Seisakusho	MA-49	
forcep without Teeth	Natsume Seisakusho	MA-48-1	
Hemostatic clips	Natsume Seisakusho	KN−353	
MicroAmp Optical 96 well reaction plate for qRT-PCR	Thermo Fisher Scientific	4316813	96-well reaction plate
MicroAmp Optical Adhesive Film	Thermo Fisher Scientific	4311971	adhesive film for 96-well reaction plate
miRNA-132-3p primer	Qiagen	MS00003458	5'UAACAGUCUACAGCCAUGGUCG
miRNA-17-5p primer	Qiagen	MS00029274	5'CAAAGUGCUUACAGUGCAGGUAG
miRNA-18a-5p primer	Qiagen	MS00031514	5'UAAGGUGCAUCUAGUGCAGAUAG
miRNA-212-3p primer	Qiagen	MS00003815	5'UAACAGUCUCCAGUCACGGCC
miRNA-21a-5p primer	Qiagen	MS00009079	5'UAGCUUAUCAGACUGAUGUUGA
miRNA-223-3p primer	Qiagen	MS00003871	5'UGUCAGUUUGUCAAAUACCCCA
miRNA-574-5p primer	Qiagen	MS00043617	5'UGAGUGUGUGUGUGUGAGUGUGU
miRNeasy Mini kit	Qiagen	217004	silica-membrane based spin column
miScript II RT kit	Qiagen	218161	
miScript SYBR Green PCR kit	Qiagen	218073	
QIA shredder	Qiagen	79654	biopolymer-shredding system in a micro centrifuge spin-column
QIAzol Lysis Reagent	Qiagen	79306	phenol/guanidine-based lysis reagent
QuantStudio 12K Flex Flex Real-Time PCR system	Thermo Fisher Scientific	4472380	real-time PCR instrument
QuantStudio 12K Flex Software version 1.2.1.	Thermo Fisher Scientific	4472380	real-time PCR instrument software
RNU6-2 primer	Qiagen	MS00033740	not disclosed
surgical scissors	Natsume Seisakusho	B-2	
Vascular clip applier	VITALITEC	1621420	

Dear Dr. Nam Nguyen:

We would like to thank you for your thoughtful review and helpful comments. We have revised our manuscript in response to your suggestions. We believe that these revisions have strengthened our manuscript and hope that it is now suitable for publication in JoVE. Point-by-point responses to your comments are provided below.

Thank you again for considering our manuscript for publication in JoVE. We look forward to hearing from you at your earliest convenience.

Sincerely,
Akinori Aomatsu

1. **Responses to Editor's Comments**

We are very grateful for the editor's thoughtful comments and suggestions.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please see the attached ithenticate report and re-write the short abstract, long abstract, highlighted portions of the introduction, protocol steps 1.4, 1.5, 1.7, 3.9, 3.14, 4.1, 5.1, 5.6, 5.7, to avoid this overlap.

→We have revised the part of the revised manuscript in accordance with these comments.

• **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 add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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).1: mention animal strain, age, sex.

→We have revised the part of the revised manuscript in accordance with these comments.

2) 1.4: unclear what extra-abdomen is.

→extra-abdominal cavity. We have revised other parts.

3) 2.1: how is blood collected? Mention needle gauge.

→Blood is collected from the inferior vena cava punctured by 27G needle.

Protocol Detail、Protocol Highlight:

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.

→ I highlight complete sentences.

References:

Commercial Language:

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

→We have revised the part of the revised manuscript in accordance with these comments.2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

→We have revised the part of the revised manuscript in accordance with these comments.

Reviewer #1:

1. Please provide functional data for showing that IRI model was made appropriately unless miRNAs confirmed are involved to only tubular necrosis.

→We make the new figure(BUN, CRE)

Minor Concerns:

1. Add surgical instruments and reagents on the table of materials.

→We have revised the part of the revised manuscript in accordance with these comments.

2. The title looks lengthy, so it would be better to reduce it. e.g. A quantitative detection method of microRNA in mouse kidney of ischemic kidney injury.

→We revised titles. There are other methods for quantifying miRNA, but is it okay to not include qRT-PCR in the title?

3. Add mouse strain and age in the protocol for IRI model.

→We have revised the part of the revised manuscript in accordance with these comments.

Reviewer #2:

Aomatsu and colleagues presented a protocol to quantify miRNAs from ischemic kidneys. While this protocol does provide some useful information, the method for RT-qPCR section was a mere summary of the manufacturer's instructions. The authors did not address the scientific

rigor and reproducibility of their protocol, which this reviewer believes are the merits of this journal: For example, the specificity and dynamic range of the qPCR assay were not discussed. These include inter- and intra-assay variation, sample number, sensitivity of the assay, linear dynamic range determination, melting temperature assay, etc., for the surveyed target miRNAs. It would be much nicer to explain a sort of guidelines for the assay used in this study. Next, the degree of kidney injury was not well determined nor developed in the protocol. Lastly, the current version of the manuscript was not well written in sound English as well as contains grammatical errors.

→We revised. In introduction, we mentioned that qRT-PCR has a wide dynamic range and accurate quantification. Troubleshooting in qRT-PCR was added to discussion.
As for creating from a primer, we are using the QIAGEN primer in this case, so I'm leaving it out of the text because it would be longer.
The mistake is often made by young people who mistake the expression of primer dimmers only for real expression. We added that you should check the Dissociation curve or Melt curve every time. The temperature range is 60-64 degrees, but it depends on the PCR devise used. Lastly, We described cycle adjustments when miRNAs are in trace amounts, but I think that will ultimately be the limit of qRT-PCR.
In such a case, We have mentioned digital PCR, which has a narrow dynamic range, but has the advantage of quantifying a small amount of genes.
The revised manuscript has been corrected by a native speaker at a language editing service. We have also checked that there are no spelling or grammatical errors in the revised manuscript.