Laser-Capture Microdissection of Renal Tubule Cells and Linear Amplification of RNA for Microarray Profiling and Real-Time PCR

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Abstract

Laser-capture microdissection (LCM) and transcriptional profiling has enabled compartment- and cell-specific analysis of gene-expression in chronic kidney disease, thus facilitating the investigation of pathophysiological associations between glomerular, tubular and interstitial structures. Due to the pico- and nanogram amounts of RNA isolated from LCM-captured material linear RNA amplification protocols are necessary prior to real-time PCR and microarray analysis. In this chapter, we describe the isolation of renal tubule cells from cryocut sections from routine kidney biopsies, and the isolation and linear amplification of RNA for downstream purposes.

Key Words: Renal, Kidney, Tubule, Microarray, Linear Amplification

1. Introduction

In the last decade a molecular-based characterization of chronic renal diseases has been proposed based on studies using novel molecular analysis tools such as microarray technology and quantitative real-time PCR (qRT-PCR)(1-2). Using these techniques several biomarkers have successfully been identified in the tubulointersitial compartment which have been associated with progression of renal injury (1). This is in line with the well known fact that prognosis of kidney disease depends more on the degree of tubulointerstitial scarring than on the degree of glomerular damage. However, gene expression between each of the compartments varies considerably (3). Since the tubulointerstitium comprises a mix of several cellular subpopulations (i.e. tubular cells, fibroblasts, leukocytes, endothelial cells), isolation of specific cells of interest (e.g. renal tubule cells) may help elucidate the mechanisms of renal disease progression in more detail (4).

Laser capture microdissection (LCM) can be applied to any tissue that is accessible through biopsy and it is used to isolate cells of interest from complex, heterogeneous cell populations with high specificity (5). Briefly, a thermoplastic transfer film is placed in contact with a 5 μm tissue section. The film is precisely activated by a 980 nm gallium-arsenide laser pulse and binds strongly to the selected cells. Then the target cells can safely be removed, and RNA can easily be isolated using various isolation methods. LCM and downstream analysis by qRT-PCR have been used successfully to determine gene expression in glomerula and specific parts of the nephron (6,7).

The limitation of LCM is the minute amount of isolated RNA which is usually in the range of picograms to nanograms. The introduction of a T-7-RNA polymerase-based linear amplification protocol enabled amplification of RNA by 1000-fold, and performing 2 rounds of this amplification protocol even allowed the analysis of the gene expression of single live neurons (8,9). Furthermore, linear amplification of RNA isolated from tubular cells has been shown to yield robust microarray results (10).

Just recently, a linear pre-amplification method using TaqMan primers and probes has been introduced for amplification of minute amounts of RNA from organ biopsies for qRT-PCR (11).

This chapter describes the isolation of renal tubule cells by LCM, the linear amplification of RNA for microarray hybridizations and the pre-amplification of RNA for qRT-PCR.

2. Materials

The protocols for tissue collection of renal biopsies suitable for LCM have recently been published in detail by others (12, 13).

2.1 Staining of renal tubule cells (RTCs) for LCM

1. Staining chambers and rack.

2. NBT staining solution: Dissolve 500 mg 4-Nitro blue tetrazolium (NBT) (Roche Applied Sciences) in 10 ml dimethylformamide (70%).
3. BCIP staining solution: Dissolve 500 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) p–toluidine salt (Roche Applied Sciences) in 10 ml dimethylformamide (100%).
4. Staining buffer: 12.11 g Trishydroxymethylaminomethane (Tris), 5.84 g NaCl, 10.17 g $MgCl_2$, RNase-free water to 1000 ml, adjust to pH 9.5 with 1 N HCl; the final buffer contains 0.1 M Tris, 0.1 M NaCl and 50 mM $MgCl_2$).
5. RNase free water.
6. 3 % glycerol.
7. Xylene.
8. 100 % ethanol.

Staining stock solutions should be protected from light and stored at +4°C.

2.2 Laser capture microdissection of renal tubule cells

1. Laser capture microscope Pixcell II™ (Arcturus, Mountain View, CA, USA).
2. CapSure™ HS LCM Caps (Arcturus, Mountain View, CA, USA).

2.3 RNA isolation from LCM-captured cells

1. PicoPure™ RNA isolation kit (Arcturus, Mountain View, CA, USA).
2. 70 % ethanol.

2.4 Linear amplification of RNA for microarray hybridization

1. Ovation® Pico WTA System (NuGEN Technologies, Inc., San Carlos, CA, USA).
2. SPRIPlate® 96R, Ring Magnet Plate (Beckman Coulter Genomics, Bernried Germany).

3. 100 % ethanol.
4. MinElute® Reaction Cleanup Kit (Qiagen, Hilden, Germany).

2.5 Pre-amplification of RNA for qRT-PCR

1. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).
2. TaqMan™ PreAmp Master Mix (Applied Biosystems, Foster City, CA, USA).
3. TaqMan™ Gene Expression Assays (Applied Biosystems, Foster City, CA, USA).
4. RNase-free water.

3. Methods

This section describes the renal tubule cell-specific alkaline phosphatase staining, LCM of tubule cells, RNA isolation and linear RNA amplification for qRT - PCR and/or microarray analysis. Tissue collection has been described in detail elsewhere (13). In brief, renal biopsies are procured using a 16-gauge needle, embedded in OCT™ (Tissue Tek™, Fisher, Pittsburgh, PA, USA), cut into 5 μm thick sections at – 20 °C using a cryomicrotome. The sections are than attached to glass slides and stored at – 80 °C.

3.1 Staining of renal tubule cells for LCM

1. Perform all staining steps at room temperature.
2. Prepare the stain by combining 100 ml staining buffer with 450 μl NBT and 350 μl BCIP stock solution. The stain is photosensitive, minimize exposure to light.
3. Place the following solutions into sterilized dye chambers:

Chamber 1: RNase-free water.

Chamber 2: 3 % glycerol.

Chamber 3: 100 % ethanol.

Chamber 4: 100 % ethanol.

Chamber 5: Xylene.

Chamber 6: Xylene.

4. Submerge sections briefly in ethanol, drain.
5. Sumberge sections in water, swirl and drain.
6. Wet sections with stain and leave for 1-2 minutes, drain.
7. Submerge sections in 3% glycerol for 20 minutes.
8. Submerge sections in ethanol, swirl and drain. Repeat in second chamber of ethanol.
9. Submerge sections in xylene for 20 minutes, drain. Repeat in second chamber.
10. Allow sections to air-dry.

3.2 Laser capture microdissection of renal tubule cells (figure)

1. Prepare a 0.5 ml microcentrifuge tube and add 50 ul Extraction Buffer (XB).
2. Place the slide with the stained sections on the microscope.
3. The tubule cells are stained violet to dark-blue. Locate and mark these target areas.
4. Place the LCM CapSure™ HS Cap onto the slide.
5. Pulse the laser. Approximately 2000 to 4000 pulses are enough to obtain 1 – 3 ng of total RNA. In our eperience a laser pulse duration of 3 ms and a laser pulse energy of 60 mW are sufficient.

6. Remove the CapSure™ HS Cap and insert it onto the prepared microcentrifuge tube.
7. Invert the microcentrifuge tube and make sure that the XB covers the complete LCM Cap.
8. Incubate the tube and the cap for 30 min at 42 °C in an incubation block.
9. Centrifuge the assembly at 800xg for 2 min to collect the extract into the microcentrifuge tube.
10. Remove the LCM cap and save the microcentrifuge tube containing the cell extract.
11. Proceed to RNA isolation or store at – 80° C.

3.3 RNA isolation from LCM-captured cells

1. For each sample prepare an RNA Purification Column by adding 250 µl Conditioning buffer to the filter membrane, incubate for 5 minutes at room temperature and centrifuge the column for 1 minute at 16,000 xg, discard the flowthrough liquid.
2. Pipette 10 µl of 70 % ethanol into the cell extract, mix well by pipetting and add to the prepared purification column.
3. Centrifuge for 2 min at 100 xg, then immediately centrifuge for 30 sec at 16,000 xg, discard flowthrough.
4. Add 100 µl Wash buffer 1 to the column and centrifuge for 1 min at 8,000 xg, discard flowthrough.
5. Add 100 µl Wash buffer 2 to the column and centrifuge for 1 min at 8,000 xg. Add another aliquot of 100 µl Wash buffer 2 and centrifuge for 2 min at 16,000 xg.

6. Transfer the column to a fresh tube and pipette 11 μl elution buffer directly onto the membrane inside the column.
7. Incubate the column for 1 min at room temperature.
8. Centrifuge the column for 1 min at 1,000 xg followed by 1 min at 16,000 xg.
9. The eluted RNA can be used immediately or stored at -80^{o}C.

3.4 Linear amplification of RNA for microarray hybridization

RNA isolated from LCM captured cells is used to generate amplified cDNA in a three-step process with the Ovation Pico WTA™ system: First-strand cDNA synthesis, DNA/RNA heteroduplex double strand cDNA synthesis and SPIA isothermal linear amplification. The amplification reaction generates cDNA with sequence complementary to the original mRNA. A minimum amount of 500 pg total RNA is required and an average 15,000-fold amplification is acheived resulting in microgram amounts of cDNA. The amplified cDNA produced is then cleaned with the MinElute™ Reaction Cleanup Kit and can undergo labeling with an appropriate dye. We use the Agilent Genomic DNA Enzymatic Labeling Kit prior to analysis with Agilent Gene Expression microarrays.

3.5.1 Synthesis of first strand cDNA

1. Thaw First strand buffer mix, First strand primer mix and water at room temperature, vortex to mix and centrifuge briefly to spin down contents. Maintain buffer mix and primer mix on ice until required. Spin down contents of First strand enzyme mix and place on ice.
2. Combine 2 μl of First strand primer mix with 5 μl total RNA (500 pg to 50 ng), incubate at 65^{o}C for 2 min then immediately place on ice.

3. Prepare a master mix containing 2.5 μl First strand buffer and 0.5 μl First strand enzyme mix. To allow for reagent that is lost during pipetting the master mix should include at least one more reaction than you have samples, 1 additional reaction for every 10 samples is usually sufficient. Add 3 μl of this master mix to each sample, mix by pipetting and spin down. Place samples into a pre-cooled thermocycler programmed as follows:

   Step 1: 4°C for 1 min

   Step 2: 25°C for 10 min

   Step 3: 42°C for 10 min

   Step 4: 70°C for 15 min and hold at 4°C

4. Once the program is complete and samples have cooled to 4°C briefly spin samples and place on ice. Proceed immediately with second strand synthesis.

3.5.2 Synthesis of second strand cDNA

1. Allow Agencourt RNAClean purification beads to warm to room temperature.
2. Thaw Second strand buffer mix, vortex briefly, spin down and place on ice. Spin down the contents of the Second strand enzyme mix and place on ice.
3. Prepare a master mix containing 9.75 μl Second strand buffer mix and 0.25 μl Second strand enzyme mix for each sample plus an additional reaction to allow for volume loss during transfer. Add 10 μl of this master mix to each sample, mix by pipetting and spin down.
4. Place samples into a pre-cooled thermocycler programmed as follows:

   Step 1: 4°C for 1 min.

   Step 2: 25°C for 10 min.

   Step 3: 50°C for 30 min.

   Step 4: 70°C for 5 min and hold at 4°C.

5. Once the program is complete and samples have cooled to 4^{o}C briefly spin samples and place at room temperature. Proceed immediately with purification of unamplified cDNA.
6. Ensure the Agencourt RNAClean purification beads have reached room temperature, resuspend beads by inverting and tapping the tube.
7. Add 32 μl of bead suspension to each sample, mix thoroughly by pipetting up and down 10 times and incubate at room temperature for 10 minutes.
8. Transfer samples to a magnet plate and stand for 5 minutes to clear the solution of beads. Carefully remove 45 ul of the binding buffer and discard.
9. With the samples still on the magnet add 200 μl of freshly prepared 70% ethanol and allow to stand for 30 seconds.
10. Carefully remove the ethanol wash with a pipette and discard.
11. Repeat this wash step two more times taking care to remove as much ethanol as possible after the final wash.
12. Air dry the beads on the magnet for 15 to 20 min then proceed immediately with the amplification step.

3.5.3 Amplification of cDNA

1. Thaw the SPIA buffer mix and the SPIA Primer mix at room temperature, vortex briefly, spin down and place on ice. Thaw the SPIA enzyme on ice, mix by inverting the tube gently 5 times (minimize bubble formation), spin briefly and place on ice.
2. Prepare a master mix by sequentially combining 80 μl SPIA buffer mix, 40 μl SPIA primer mix and 40 μl SPIA enzyme mix per sample. Add 160 μl of this master mix to the samples bound to the dried beads. Use a pipette to mix each sample well

and resuspend the beads. Transfer one half of ech sample (80 μl) into a second tube.

3. Place samples in a pre-cooled thermal cycler programmed as follows:

   Step 1: 4°C for 1 min.

   Step 2: 47°C for 60 min.

   Step 3: 95°C for 5 min, and hold at 4°C.

4. Once the program is complete and samples have cooled to 4°C briefly spin samples and place on ice. Re-combine the half reactions.
5. Transfer samples to a magnet plate for 5 min to clear the solutions of beads. Carefully remove all of the supernatant containing the eluted cDNA and transfer to a fresh tube.
6. At this stage the cDNA can be stored at -20°C or purified using the MinElute™ Reaction Cleanup Kit. Clean cDNA is ready to undergo labeling and hybridization to the oligonucleotide microarray platform of choice.

3.6 Pre-amplification of RNA for qRT-PCR

First, RNA is converted to single-stranded cDNA by a reverse transcription enzyme. Then, the preamplification process selectively amplifies a cohort of genes in a linear manner so that an expression profile for the selected genes may be determined from a small sample of cDNA. The protocol can be adapted for either 1,000-fold (10 cycles) or 16,000-fold (14 cycles) amplification of the selected genes. Before proceeding with preamplification of cDNA from the limited samples it is vital to check the TaqMan assays for preamplification uniformity using a control cDNA from a non-limited source.

3.6.1 Reverse Transcription Reaction

1. Thaw High-Capacity cDNA Reverse Transcription kit reagents and RNA samples on ice.
2. To allow for reagent that is lost during pipetting the master mix should include at least one more reaction than you have samples, 1 additional reaction for every 10 samples is usually sufficient. Prepare a 2X master mix using the following quantites of reagent per reaction, mix gently and maintain on ice:

   2 μl 10X RT Buffer.

   0.8 μl 25X dNTP Mix (100 mM).

   2 μl 10X RT random primers.

   1 μl MultiScribe™ Reverse Transcriptase.

   1 μl RNase Inhibitor and 3.2 ul nuclease-free water.
3. Pipette 10 μl RNA into a PCR tube and add 10 μl master mix to the sample, mix by gentle pipetting and seal the tube. If necessary briefly centrifuge the samples to spin down contents to the bottom of the PCR tubes, maintain on ice until ready to transfer to the thermal cycler.
4. Create the following program in the thermal cycler you will use:

   Step 1: 25^{o}C for 10 min.

   Step 2: 37^{o}C for 120 min.

   Step 3: 85^{o}C for 5 sec.

   Step 4: 4^{o}C hold.
5. Load the samples into the thermal cycler and begin the program.
6. Once the cDNA samples have cooled remove them from the thermal cycler. The cDNA may be transferred to a RNase-free microcentrifuge tube and stored at -20^{o}C until required or used immediately.

3.6.2 Preamplification reaction

1. Prepare a pooled assay mix of the TaqMan gene expression assays for the genes of interest, a maximum of 100 genes can be preamplified. Thaw the 20X assays on ice and pipette 5 μl of each assay into a nuclease-free microcentrifuge tube. Add 1X TE buffer to a final volume of 500 μl. For example, if the pooled assay mix contains 5 μl each of 10 assays add 450 μl TE buffer for a final volume of 500 μl. Each assay is present at a final concentration of 0.2X. The pooled assay mix can be frozen at -20^{o}C for storage or used immediately.
2. Pipette 1-250 ng cDNA sample into a PCR tube, aim to use a consistent amount for all samples, and adjust the volume with nuclease-free water to 10 μl. Add 10 μl pooled assay mix and 20 μl TaqMan® PreAmp Master Mix to each sample, mix gently by pipetting and maintain on ice until ready to transfer to the thermal cycler.
3. Create the following program in the thermal cycler:

   Step 1: 95^{o}C for 10 min

   Step 2: 10 cycles at 95^{o}C for 15 sec

   Step 3: 60^{o}C for 4 min
4. Load the samples into the thermal cycler and begin the program. Once cycling is complete it is important to immediately transfer the samples from the cycler onto ice to halt the reaction.
5. Transfer the preamplified sample to a nuclease-free microcentrifuge tube and dilute with 160 μl 1X TE buffer. The preamplified cDNA can be stored at -20^{o}C or used immediately in a PCR reaction.

4. Notes

1. When more than 5000 LCM pulses are performed we increase the laser energy to 80 mW. For glomeruli longer durations than 3 ms may be needed, e.g. 10 ms. We always take the smallest of the three sizes of the laser beam. Try to focus the laser beam exactly to the level of the section placed on the slide. The microdissection works best when you allow the cap to warm up for 2 – 3 min.
2. RNA quality control can be performed using Agilent Bioanalyzer RNA Pico Chips™ (both Agilent Technologies, Palo Alto, CA, USA). However, due to the minute amounts of RNA we rarely perform this quality control step. We rather measure the expression values of certain housekeeper genes such as cyclophilin A (PPIA).
3. Although recommended by others (13) we do not perform a DNase step since we do not experience contamination by DNA and we observe a considerable degree of RNA degradation during this treatment.
4. In our experience 90% of target genes show uniform and reproducible preamplification using the TaqMan™ PreAmp protocol, which is also reported by Applied Biosystems. Since the reason for a reliable linear pre-amplification is not entirely clear we strongly recommend testing the linearity of each assay on non-limited RNA. There have been published reports of frequently used housekeeping genes such as HPRT (11) and GAPDH (14) not attaining ΔΔCt values within the +/- 1.5 range. The endogenous uniformity reference recommended by Applied Biosystems is CDKN1B (Assay ID Hs00153277_m1). In kidney tissue we have found PPIA (Assay ID Hs99999904_m1) to be a reliable reference gene.
5. The ovation™ Pico WTA System (NuGen) works best with RNA amounts > 1 ng.

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Figure. Cryosection of renal tubule cells from a routine cryocut kidney biopsy stained for alkaline phosphatase. **A** After NBT/BCIP staining alkaline phosphatase in the brush border of the tubule cells stain violet to dark blue. **B, C** The stained tubule cells are targeted, laser captured and adhered to the transfer film. **D** Kidney biopsy section after LCM of renal tubule cells. Magnification x 100.