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Analysis of Asbestos-Induced Gene Expression Changes in Bronchiolar Epithelial Cells Using Laser Capture Microdissection and Quantitative Reverse Transcriptase–Polymerase Chain Reaction

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Summary

Laser capture microdissection (LCM) enables the removal of discrete microstructures or cell types from properly prepared histological sections. Extraction of RNA from microdissected tissue followed by quantitative reverse transcriptase–polymerase chain (QRT-PCR) reaction permits the analysis of cell-type or microstructure-specific gene expression changes that occur in response to various stimuli in the environment. In our lab, the combination of LCM and QRT-PCR has proven very useful in the determination of the in vivo gene expression changes that occur in bronchiolar epithelium in response to inhalation of crocidolite asbestos. A detailed description of the preparation of cDNA from bronchiolar epithelial cells obtained by LCM is described in this work.

Key Words: Asbestos; bronchiolar epithelium; environmental pathology; laser capture microdissection; lung disease; microgenomics; quantitative reverse transcriptase–polymerase chain reaction.

1. Introduction

Laser capture microdissection (LCM) is a relatively new and unique technique of increasing popularity that facilitates the identification of cell-type or microstructure-specific changes in gene expression by providing a means to selectively remove individual cells or small groups of cells from properly prepared tissue sections *(1,2)*. The microdissection instrument that has been most widely used, the Arcturus PixCell II, relies on a polymer film that is placed over the tissue of interest. A short-duration pulse from an infrared laser is used to select the desired cells by melting the area of the film with which they are in

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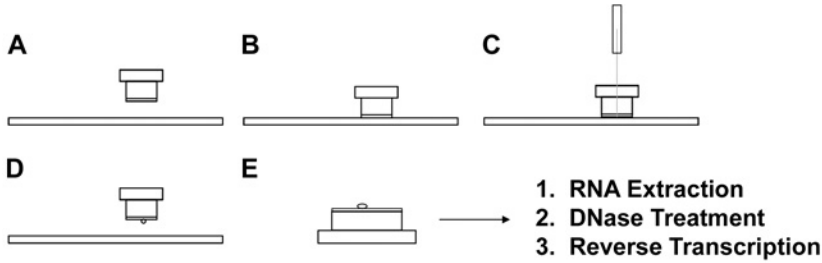

Fig. 1. Laser capture microdissection and RT–PCR. **(A)** Dehydrated tissue section and cap with polymer film on surface facing slide. **(B)** The cap is placed over the tissue to be microdissected. **(C)** An infrared laser pulse is directed at the film in contact with the cells of interest. **(D)** Removal of the cap after the film is melted also removes the microdissected cells. **(E)** The microdissected cells can be used for analysis of gene expression using RT-PCR.

contact. RNA can then be extracted from these cells for quantitation via quantitative reverse transcriptase–polymerase chain reaction (QRT-PCR) or qualitative assessment for the presence or absence of expression of a gene by gel-based RT-PCR.

One of the challenges facing researchers interested in understanding the in vivo signaling mechanisms and changes in gene expression involved in the responses of the lung to environmental insults, such as asbestos, is its complex architecture. The lung is a highly heterogeneous organ with over 40 cell types. An important consequence of this heterogeneity is that the responses of individual cell types to pollutants are diluted in measurements of gene expression in relatively crude preparations such as whole-lung extracts. The combination of LCM and QRT-PCR (*see* **Fig. 1**) is particularly useful for measuring changes in expression of transcription factors and markers of proliferation and injury in lung epithelial cells following exposure to asbestos ***(3)***. In this chapter, the methodology for the isolation of DNA-free RNA from bronchiolar epithelial cells (*see* **Fig. 2**) and subsequent preparation of cDNA are described in detail ***(4–6)***.

2. Materials

2.1. Kits and Reagents

1. Arcturus PicoPure RNA isolation kit: Components include conditioning buffer (CB), extraction buffer (XB), 70% ethanol (EtOH), wash buffer 1 (W1), wash buffer 2 (W2), elution buffer (EB), and RNA purification columns.
2. Ethanol (ETOH) series dilutions (70% and 95% ETOH): Add 30 or 5 mL of autoclaved distilled-deionized water (ddH_20) to prepare. 70 or 95 mL of 100% ETOH to 100 mL.
3. Arcturus CapSure LCM caps, CapSure pads, and prep strips (included with caps).

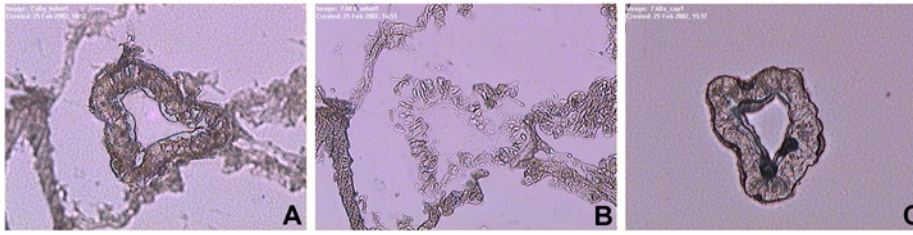

Fig. 2. Removal of a bronchiole from a lung section using laser capture microdissection: before microdissection **(A)**, after microdissection **(B)**, and the isolated bronchiole on the surface of the cap **(C)**.

4. Promega RQ1 RNase-free DNase.
5. Promega Reverse Transcription system.

2.2. Equipment

1. A cryostat suitable for cutting 10-µm thick sections from tissue embedded in optical cutting temperature (OCT) at –20°C is required for generating sections that can be properly microdissected.
2. An Arcturus PixCell II laser capture microdissector is used to remove bronchiolar epithelium from prepared sections.
3. A dessicator, preferably with a vacuum pump, is needed to keep the prepared tissue sections dehydrated until use.

3. Methods

3.1. Specimen Preparation

Following euthanasia by a combination of 5 mg sodium pentobarbital and pneumothorax, the lungs are inflated with calcium/magnesium-free phosphate-buffered saline (PBS) to a pressure of 30 cm H_2O. After occluding the primary bronchi by tying off with surgical suture, one lung is immersed in a cryomold containing OCT embedding compound. The cryomold is then dipped into liquid-nitrogen-cooled isopentane until the appearance of the OCT changes from clear to opaque. Immediately remove the new specimen block and transfer to a –80°C freezer.

3.2. Slide Preparation

3.2.1. Sectioning

Specimen OCT blocks should be equilibrated at –20°C for 20 min prior to sectioning. Sections should be cut at 10 µm and transferred within min to a –80°C freezer for storage or processed immediately for microdissection. The sections must be kept cold to minimize RNA degradation in the tissue of interest (*see* **Notes 1** and **2**).

3.2.2. Dehydration

It is essential that tissue be completely dehydrated for efficient transfer of cells from the tissue section to the polymer film on the CapSure caps. The following procedure should result in tissue ready for microdissection (typically done in groups of 5–10 slides with Coplin jars):

1. Immerse in cold 70% ETOH (chilled to –20°C) for 60 s.
2. Immerse in ddH_20 for 30 s.
3. Immerse in 70% ETOH for 60 s.
4. Immerse in 95% ETOH for 60 s.
5. Immerse in 100% ETOH for 2 min.
6. Immerse in xylene twice for 10 min each.
7. After removing sections from xylene, allow them to dry for 5 min in a fume hood.
8. Keep slides in a vacuum dessicator until use (for no more than 12 h).

3.3. Microdissection

1. Prior to microdissection, weakly adhering tissue should be removed from the slide using the prep strips included with the CapSure caps. This is accomplished by peeling off the backing of the prep strip and pressing it onto the section. Next, apply pressure on the strip with two fingers and move along the strip in one direction, taking care not to apply so much force as to damage the tissue on the slide.
2. For bronchiolar epithelium, a 7.5-µm laser spot size is best to achieve capture. The power and duration of laser pulses should be adjusted as necessary to get good capture of the desired cells. To test if the settings are correct, position the laser over the lumen of a bronchiole and pulse the laser. If the settings are correct, the laser will leave a dark ring with a clear center in the film.
3. After collecting the cells of interest onto a cap, gently roll the film surface of the cap over the exposed surface of a CapSure pad. This will remove tissue that was not selected but adhered to the cap nonspecifically.
4. Immediately snap the cap into the opening of a 0.5-mL RNase-free Eppendorf tube that contains 50 µL of extraction buffer. Invert the tube and, in that position, incubate at 42°C for 30 min.
5. Centrifuge at 800*g* for 2 min to pull the extraction buffer to the bottom of the tube. Pull off the cap and close the lid to the tube. Immediately snap-freeze the extract using dry ice and place in a freezer at –80°C for storage. If desired, the rest of the isolation can be performed immediately.
6. Apply 250 µL of conditioning buffer to the surface of the filter in the purification columns. Allow the column to equilibrate for 5 min at room temperature. After the column is equilibrated, centrifuge at 16,000*g* for 1 min.
7. Thaw the samples and pipet 50 µL of 70% ethanol (from the kit) to each sample. Mix well using the pipet.
8. Add the mixture of ethanol and extract to the purification column and centrifuge for 2 min at 100*g*, followed by 30 s at 16,000*g*.

9. Add 100 µL of W1 to each column and centrifuge at 8000*g* for 1 min.
10. Add 100 µL of W2 to each column and centrifuge at 8000*g* for 1 min.
11. Add another 100 µL of W2 to each column and centrifuge at 16,000*g* for 1 min.
12. Remove all filtrate from the tube and replace the purification filter. Centrifuge at 16,000*g* for 1 min to ensure that the filter is dry.
13. Transfer the purification filter to a new tube (from the kit) and elute by applying 24 µL of elution buffer to the filter, allowing equilibration for 1 min, and centrifuging for 1 min at 16,000*g*.

3.4. DNase Treatment

1. Add 3 µL of Promega RQ1 DNase 10X reaction buffer to each sample.
2. Add 3 µL of RQ1 RNase-Free DNase to each sample.
3. Incubate at 37°C for 30 min.
4. Add 3 µL of RQ1 DNase stop solution to each sample.
5. Incubate at 65°C for 10 min.

3.5. Reverse Transcription

To each sample add the following reagents from the Promega Reverse Transcription kit and follow **steps 6–13**:

1. 12 µL of 25 m*M* $MgCl_2$.
2. 6 µL of 10X reverse transcription buffer.
3. 6 µL of 10 m*M* dNTP mixture.
4. 1.5 µL of Rnasin.
5. 3 µL of random hexamers.
6. Mix by pipetting.
7. Remove 10 µL from each sample to serve as that sample's "no reverse transcription" (NRT) control.
8. Add 45 U AMV reverse transcriptase to each sample and 1 µL of nuclease-free water to each NRT control.
9. Incubate all tubes at room temperature for 10 min.
10. Incubate all tubes at 42°C for 1 h.
11. Incubate all tubes at 95°C for 5 min.
12. Cool tubes on ice for 5 min.
13. Freeze tubes at –20°C. The cDNA is now ready for analysis using either Q-PCR or gel-based PCR.

4. Notes

1. Sectioning should be performed as quickly as possible to preserve the integrity of the RNA within specimen blocks.
2. As with any technique involving RNA, special care must be employed to prevent RNase contamination. Gloves should be changed regularly. Benchtops should be cleaned with an RNase-destroyed agent such as RnaseZap from Ambion. The surfaces of the cryostat should also be cleaned prior to sectioning. RNase-free filter tips for pipets and RNase-free tubes are also essential.

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