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Epigenetic Analysis of Laser Capture Microdissected Fetal Epithelia¹

Ratnam S. Seelan^a, Dennis R. Warner^a, Partha M. Mukhopadhyay^a, Sarah A. Andres^b, Irina A. Smolenkova^a, James L. Wittliff^b, M. Michele Pisano^{a,*}, and Robert M. Greene^a

^aBirth Defects Center, Department of Molecular, Cellular and Craniofacial Biology, ULSD, University of Louisville, 501 S. Preston St., Louisville, KY - 40202

^bDepartment of Biochemistry & Molecular Biology and Institute for Molecular Diversity & Drug Design, University of Louisville, 319 Abraham Flexner Way, Louisville, KY - 40202

Abstract

Laser capture microdissection (LCM) is a superior method for non-destructive collection of specific cell populations from tissue sections. While DNA, RNA and protein have been analyzed from LCM-procured samples, epigenetic analyses, particularly of fetal, highly hydrated tissue, have not been attempted. A standardized protocol with quality assurance measures was established to procure cells by LCM of the medial edge epithelia (MEE) of the fetal palatal processes for isolation of intact microRNA for expression analyses and genomic DNA for CpG methylation analyses. MicroRNA preparations, obtained using the RNAqueous[®] Micro kit (Life Technologies), exhibited better yields and higher quality than those obtained using the Arcturus[®] PicoPure[®] RNA Isolation kit (Life Technologies). The approach was validated using real-time PCR to determine expression of selected microRNAs (miR-99a and miR-200b) and pyrosequencing to determine CpG methylation status of selected genes (*Aph1a* and *Dkk4*) in the MEE. These studies describe an optimized approach for employing LCM of epithelial cells from fresh frozen fetal tissue that enables quantitative analyses of miRNA expression levels and CpG methylation.

Introduction

Isolation of homogenous populations of cells by laser capture microdissection (LCM) provides an effective technique with which to examine gene regulatory mechanisms operative in heterogeneous adult and fetal tissue [1–3]. As such, it has been used effectively

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*Corresponding author: M. Michele Pisano, Ph.D., Department of Molecular, Cellular and Craniofacial Biology, ULSD, Birth Defects Center, University of Louisville, 501 S. Preston Street, Suite 350, Louisville, KY 40202, Phone: (502) 852-1962, Fax: (502) 852-4702, pisano@louisville.edu.

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to isolate DNA, RNA or protein from specific cell-types in heterogenous tissue sections [4]. However, little information exists on whether LCM may be used to discern the expression of microRNAs, or the CpG methylation status of genes in LCM-procured cells of fresh frozen, highly hydrated, fetal tissues. The present study tests the feasibility of this approach and establishes a standardized protocol using the medial edge epithelia (MEE) of the murine secondary palate as a target tissue.

The MEE is a transient component of the developing secondary palate (roof of the oral cavity). This structure develops between gestational days (GDs) 12–14 in the mouse, originating as a pair of oral projections (palatal processes) from the maxillary processes of each first brachial arch. These palatal processes reorient to assume a position above the tongue and fuse with each other to separate the oral and nasal cavities [5,6]. Fusion of the two palatal processes involves a complex sequence of differentiated steps involving epithelial mesenchymal transition (EMT), apoptosis and/or cell migration of the MEE of each palatal process [5,7–11]. While substantial progress has been made in identifying the salient genes/proteins regulating development of the palate, little headway has been made in understanding the role of epigenetics in this process, particularly as it relates to MEE differentiation. This is due, in part, to the difficulty in isolating a homogenous population of epithelial cells of sufficient quantity and quality from within the heterogeneous fetal palatal tissue to enable epigenetic analyses. We thus describe an optimized approach with stringent quality assurance measures for isolation of epithelial cells from fresh frozen fetal tissue that enables quantitative analyses of miRNA expression levels and CpG methylation. Our protocol was validated by real-time PCR of selected microRNAs expressed in the MEE and by pyrosequencing analysis of selected genes in the MEE.

Materials and Methods

Animals

Mice used in this study were of the Hsd:ICR strain (CD-1) and were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). The use of this strain was approved by the University of Louisville Institutional Animal Care and Use Committee. Mice were housed in an American Association for Accreditation of Laboratory Animal Care approved facility. The animals were provided food and water *ad libitum* and exposed to a 12h on/off light cycle. To obtain timed matings, a single mature male was housed overnight with two mature, nulliparous females and the presence of a vaginal plug the following morning was taken as evidence of mating and considered as GD 0.5.

Sample preparation

Appropriate care was taken for proper tissue procurement, specimen handling and cryopreservation as previously described [12]. Pregnant mice of the appropriate gestation age (GD 13.0 – 14.5) were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. Fetuses were immediately removed by caesarian section and placed into ice-cold PBS. Fetuses were mounted in O.C.T. embedding medium (Sakura Finetek USA, Torrance, CA), snap-frozen in an ethanol/dry-ice bath and stored at -80°C , until sections were collected. O.C.T. blocks were equilibrated to -20°C and then mounted in a Leica

CM1900 Cryostat (Leica Microsystems Inc., Buffalo Grove, IL). The chuck and cabin temperatures were maintained at -20°C . A low-profile disposable blade was used (Leica Microsystems, Inc.). Fetuses were sectioned in the coronal plane to a thickness of $7\text{ }\mu\text{m}$ and collected onto Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) - two sections/slide - and allowed to freeze at -20°C for 5 min then placed at -80°C .

Laser Capture Microdissection (LCM)

Prior to analyses, the structural integrity of frozen tissue sections was evaluated after hematoxylin and eosin (H & E) staining using a modified protocol [1,12–14] stressing reduced time in order to minimize RNA degradation, while adequately staining sections for visualization of cell types without a cover slip. Tissue sections were stained with H & E, and nucleic acids were extracted from intact sections scraped off slides using the same methods employed for LCM-procured cells to ensure that there was no difference in the extent of nucleic acid degradation attributable to H & E staining [1,12–14]. Cells of the palatal MEE were laser captured using the PixCell IIeTM (Applied Biosystems/Life Technologies, Carlsbad, CA) in CapSureTM HS LCM Caps (Arcturus/Life Technologies). This permitted the non-destructive collection of intact cells on the surface transfer film of the cap. Typically, MEE cells from each frozen tissue section were collected using 50–175 laser pulses ($7.5\text{ }\mu\text{m}$ “spot” size) within 30 min of H & E staining. A fixation protocol using ice-cold acetone/75% ethanol (1:1) for 2 min, followed by treatment with 75% ethanol for 2 min, 95% ethanol for 15 sec, 100% ethanol for 5 sec, and xylene for 4.5 min (www.facebase.org/node/154) did not substantially improve RNA yields or quality. This was inferred from the observation that *18S* and *Tgfb3* expression levels in tissue scrapes or LCM-procured cells after fixation were virtually identical to those from unfixed tissues when assayed by real-time PCR (discussed below). Thus, a fixation step was omitted from the protocol.

Isolation and characterization of RNA from LCM-procured cells

LCM-procured MEE cells from 15–30 sections of palate tissue at a single stage of differentiation were pooled and total RNA was extracted by one of two methods which were both optimized for the small numbers of cells procured by LCM. RNA was isolated using either: 1) the Arcturus[®] PicoPure[®] RNA Isolation kit (Applied Biosystems/Life Technologies) with an on-column DNase I digestion, or 2) the RNAqueous[®] Micro kit (Ambion/Life Technologies) including a DNase I digestion after elution. Extracted RNA was evaluated utilizing the Agilent RNA 6000 Pico Kit (Agilent Technologies, Palo Alto, CA) and BioanalyzerTM (Agilent Technologies). For comparative analysis, total RNA from an intact tissue (whole palate) was isolated using the RNeasy kit (Qiagen).

Isolation of genomic DNA from LCM-procured cells

LCM-procured MEE cells from 10–18 sections of palate tissue at a single stage of differentiation were pooled. gDNA was extracted using the Arcturus[®] PicoPure[®] DNA Extraction kit (Applied Biosystems/Life Technologies). DNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

Determination of microRNA levels from LCM-procured cells

To synthesize cDNA from microRNA, the TaqMan® MicroRNA Reverse Transcription kit was used (Life Technologies) with pooled primers (Megaplex™ Rodent Pool A; Life Technologies). Due to the low number of cells in the MEE collected by LCM, a pre-amplification step was used, following cDNA synthesis and before real-time PCR. A matched pool of pre-amplification primers combined with a TaqMan PreAMP master mix (Life Technologies) was used for this step, as described by the manufacturer. Real-time PCR was undertaken on a TaqMan ViiA7 real-time PCR instrument with probe/primer sets to specific microRNAs (Life Technologies). Small/microRNAs from an intact tissue (medial nasal process, MNP) were isolated using the mirVana kit (Ambion) for comparative analysis.

Pyrosequencing of genomic DNA obtained from LCM-procured cells

Six genes, found to be methylated during the process of palatal fusion in the mouse [15], were selected for determining CpG methylation levels by pyrosequencing. About 1 µg gDNA was bisulfite-modified using the EpiTect Bisulfite kit (Qiagen, Valencia, CA) and purified in a total volume of 40 µl of elution buffer, following the low concentration DNA protocol. One µl of the eluted DNA was amplified using the PyroMark PCR kit (Qiagen) in a 25 µl total volume and CpG methylation levels were assessed using 10 µl of the amplified product in a PyroMark Q24 system (Qiagen). Primers for the amplification of genes from bisulfite-modified DNA were custom designed using the PyroMark Assay Design 2.0 software (Qiagen).

Results and Discussion

There are a limited number of reports that describe analysis of microRNA expression and gene-specific methylation in LCM-acquired samples. A PubMed search for LCM and microRNAs in the National Center of Biotechnology Information (NCBI) database retrieved 12 reports [16–27] (Supplemental Table 1), all but one [20], describing the use of fixed tissue samples; in contrast to our protocol, this report [20] identified differentially expressed miRNAs from normal and epithelial tumor cells of urothelial cell carcinoma after LCM and deep-sequencing of small RNA libraries (see also below). For gDNA isolation, a search for LCM and DNA Methylation/CpG Methylation/Pyrosequencing retrieved 21 reports [17, 21, 28–46], 5 of which determined gene-specific CpG methylation levels by bisulfite sequencing analysis (Supplemental Table 1). Only one report described the use of pyrosequencing to analyze methylated CpG residues in LCM-procured gDNA [17]; however, this was a step by step protocol for using fixed tissue samples and provided no experimental results for data evaluation. One of the aims in developing this protocol was also to examine if gDNA obtained from fresh frozen, LCM-procured cells was amenable to pyrosequencing. To generate a successful pyrogram and a reliable quantitation of CpG methylation levels, an unambiguous amplification and adequate quantity of the PCR product must be obtained from bisulfite-modified gDNA. The optimized protocol described here, therefore, differs substantially from all of the above mentioned studies in that fresh frozen fetal tissue - the MEE of the developing secondary palate - was obtained and processed without fixation for miRNA and pyrosequencing analyses. The protocol is rapid resulting in

high quality microRNAs/mRNAs and gDNAs suitable for analyzing gene expression in specific cell types. Typical yields of RNA and DNA and the quality of RNAs obtained using the LCM protocol are presented in Table 1. It should be noted that the isolation of MEE of palatal tissues by LCM has been reported before, but these studies pertained to the analysis of mRNA expression using fixed tissues [47, 48].

RNA lability is the major concern when using LCM for RNA-based analyses. RNA degradation ensues due to several factors, but the primary reasons appear to be the additional time taken to process tissues (fixation, staining, laser capture), which increases the risk of hydrolysis of the thermodynamically unstable RNA molecules and/or exposes them to contaminating ribonucleases [13,47]. A number of fixation methods were initially utilized but these did not substantially improve RNA quality, or yields, when compared to unfixed fresh frozen tissues. A result of a typical experiment is shown in Table 2 where RNA quality and yields were assessed by determining 18S rRNA levels in fixed and unfixed tissue scrapes and LCM-procured tissues. Comparison of Ct values indicate that fixation did not significantly enhance RNA quality/yields. Thus, a fixation step was omitted from the LCM protocol. Indeed, it is well known that the use of fixatives often compromises RNA quality [49, 50]. The optimized LCM protocol takes, on average, ~30 minutes to process a slide containing 2 tissue sections, from the time of its retrieval from storage (−80°C) to LCM tissue isolation. A more extensive discussion on the precautions necessary when attempting tissue procurement by LCM for RNA analysis is presented elsewhere [2,13]. The structural integrity of the frozen tissue was maintained throughout the period of LCM, permitting the collection of intact cells within 30 minutes of H & E staining (Figure 1).

Two kits were tested for their effectiveness in microRNA extraction (Arcturus® PicoPure® RNA Isolation kit and the RNAqueous® Micro kit; both from Life Technologies). Yield and quality of the isolated RNAs were assessed. For optimization of the protocol, experiments were initially carried out with RNAs extracted from tissue scrapes. Optimized methods were then applied to RNAs derived from LCM-procured cells. RNA quality was assessed by RNA sizing, using the Agilent Bioanalyzer, and real-time PCR of small/microRNAs. The initial steps preceding LCM (fetal preparation and sectioning) preserved RNA integrity, as Bioanalyzer scans of RNAs extracted from tissue scrapes typically indicated a RIN (RNA Integrity Number) [51] >9 (data not shown).

Based on RNA yield and raw Ct values of selected small/microRNAs, the RNAqueous® Micro kit was somewhat better than the PicoPure® kit in its ability to extract small/microRNAs (Table 3) and mRNAs (Table 4). The RNAqueous® protocol yielded ~ 9-fold more RNA from scraped cells than those obtained from the PicoPure® kit (data not shown). For evaluating microRNA expression, sno-135 (a 57 nt small nucleolar RNA commonly used for normalizing microRNA levels) and two microRNAs with essential roles in palatogenesis (miR-99a and miR-200b) were examined. miR99a modulates TGF-β induced EMT [52] whereas miR-200b, which is strongly expressed in palatal epithelium prior to fusion of palatal processes, regulates cell migration during palate development [53]. For scraped tissue samples, the RNAqueous® Micro kit yielded lower Ct values (indicative of higher expression or higher yields) for all three small/microRNAs tested, compared to that from the PicoPure® kit; notably, a ~ 6–9 fold difference in Ct values was observed with

these microRNAs (Table 3). For LCM samples, nearly identical Ct values were obtained for sno-135 and miR-99a samples, but miR-200b had higher expression/yields with the RNAqueous® Micro kit (36.51 ± 0.81) than with the PicoPure® kit (39.53 ± 0.92). When Ct values of scraped and LCM-procured samples were compared to those isolated directly from an intact tissue (medial nasal process; MNP), the latter exhibited slightly lower average Ct values (Table 3), suggesting some level of RNA degradation or reduced yields of small/microRNAs extracted from tissue scrapes and LCM-procured samples.

Both kits were also used to evaluate extracted levels of two mRNAs expressed in the MEE: *Mmp13* and *Tgfb3* (Table 4). *Mmp13* is almost exclusively localized in both epithelial and mesenchymal cells at the site of palatal fusion [54], whereas, *Tgfb3* is specifically expressed in cells of the MEE during palatal fusion [55, 56] as well as in the oral and nasal epithelia of palatal tissue. The RNAqueous® Micro kit was considerably more efficient in extracting *Mmp-13*, but not *Tgfb3*, mRNA from both scraped and LCM-procured tissue. 18S rRNA control levels were similar between the two kits when used for scraped or LCM-procured tissue. As noted earlier, LCM samples had relatively higher Ct values than scraped samples. The level of *Mmp13* mRNA expression observed in RNA isolated directly from whole palate tissues was below the level of detectability (Ct value >40) and is most likely due to sample dilution – i.e., *Mmp13* mRNA localized to epithelial and mesenchymal cells at the site of palatal fusion [54, 57] is likely to have been diluted when the entire palate was extracted for RNA. This highlights the utility of using LCM rather than whole tissue samples for cell-specific gene expression analysis. Overall, these observations indicate that the performance of the RNAqueous® Micro kit is much superior to the PicoPure® kit, especially as it pertains to small/microRNA isolation. Consistent with this is the observation by Goossens et al [18] that microRNAs were not retained using the PicoPure® kit, but is in contrast to the report by Chen et al [20] who used the PicoPure® kit to purify their small RNAs. The RNAqueous® Micro kit was thus used for all subsequent analyses. Representative Bioanalyzer scans of RNA obtained from LCM-procured cells of the MEE (from GDs 13.5, 14.0, and 14.5), and isolated using the RNAqueous® Micro kit are shown in Figure 2. RINs ranged from 8.0 – 9.2, indicating the high quality of the isolated RNAs. A representative amplification plot of small RNA controls from a preparation of LCM-procured MEE cells displayed the typical exponential and plateau phases of a successful amplification (Figure 3).

gDNAs from LCM-procured tissue were isolated using the Arcturus® PicoPure® DNA Extraction kit. Six genes were chosen for CpG methylation analysis based on an ongoing study that identified them as being methylated during palate development [15]. Of the six genes, four were effectively amplified by PCR of bisulfite-modified LCM-procured DNA (Figure 4A, left panel) prior to pyrosequencing. Failure of two of these genes (*Dkk4* and *Ctnnb1*) to amplify may have been the result of gDNA fragmentation. This fragmentation is unlikely to be due to the heat generated by the laser beam [1–3] as a low power infra-red laser was used [4]; rather, degradation may be due to the harsh chemical conditions that the LCM-derived gDNA was subjected to during bisulfite modification. Nevertheless, in a subsequent experiment using fresh bisulfite-modified LCM DNA, *Dkk4* was successfully PCR amplified (Figure 4A, right panel). Representative pyrograms generated from the

amplification products of *Aph1a* and *Dkk4* are shown in Figure 4B. To assess the success rate of PCR amplification of genes for pyrosequencing, a set of 4 genes was amplified from 4 individually prepared bisulfite-modified gDNAs (derived from two independent LCM-procured MEE samples). Our results demonstrated the amplification of 3 different genes from each of three bisulfite modified gDNAs and all genes were amplifiable from at least one bisulfite-modified gDNA. The PCR success rate was calculated to be 62.5%, on average.

In conclusion, we have established a reproducible protocol with quality control measures for the preparation of small/microRNAs and gDNAs of very high quality from specific cell types procured by LCM of fresh frozen unfixed fetal tissue. The RNAqueous[®] Micro kit was found to be superior to the PicoPure[®] RNA Isolation kit in its ability to extract small/microRNAs of high quality and yields. Although, the use of LCM resulted in RNA preparations exhibiting relatively higher Ct values compared to that isolated from intact tissues, the quality of RNAs was excellent, with RIN numbers typically >8.0. The methylation status of representative CpG residues of genes could also be discerned by pyrosequencing of the bisulfite-modified gDNA extracted from LCM-procured cells. This optimized protocol should prove exceedingly helpful in epigenetic analyses of specific cell types from fresh frozen fetal tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Laser capture microdissection (LCM) of medial edge epithelia (MEE) from GD13.5, GD14 and GD14.5 secondary palates

LCM of MEE from GD13.5 (Panels A–D), GD14 (Panels E–H) and GD14.5 (Panels I–L) is shown. Panels A, E and I in each row depict a representative frozen tissue section indicating tissue integrity, prior to LCM of cells (4X magnification). The remaining three panels, in each row, respectively, show the MEE before (B, F, and J), and after LCM (C, G, and K), and the MEE collected onto the LCM cap for subsequent DNA or RNA analysis (D, H, and L) (20X magnification). The arrows indicate the MEE region that was microdissected. MEE, medial edge epithelium; PS, palatal shelf; T, tongue.

Figure 2. Bioanalyzer scans for LCM-procured MEE RNAs using the RNAqueous® Micro kit Representative Bioanalyzer scans of RNAs from LCM-procured MEE from GD13.5 (A), GD14.0 (B) and GD14.5 (C) secondary palates obtained using the RNAqueous® Micro kit. The RIN (RNA Integrity Number) for these RNAs (9.2, 8.0 and 8.6, respectively) indicates the high quality of the RNAs isolated from LCM-procured cells.

Figure 3. Representative amplification plot of control small RNAs

cDNAs were synthesized from RNA purified from LCM-procured MEE cells from GD14.0 palatal shelves and analyzed for the expression of control, small RNAs by semi-quantitative real-time PCR. The graph shows ΔR_n values as a function of cycle number for U6 (4 wells), U87, snoRNA-135 and snoRNA-202 small nucleolar RNAs, Y1 non-coding RNA, and ath-miR159a (a microRNA expressed in Arabidopsis, used as a negative control). Note: R_n is the signal from the FAM fluorescent dye (reporter) normalized to that for ROX (internal control). These data demonstrate efficient PCR-amplification of all small RNAs tested, with the exception of the negative control (ath-miR159a). When reverse transcriptase was omitted from the cDNA synthesis reaction, no amplification of positive control RNAs was detected (data not shown). RNAs were isolated using the RNAqueous[®] Micro kit.

Figure 4. CpG methylation analysis by pyrosequencing
A, left panel. Six genes were selected for PCR amplification of bisulfite-modified genomic DNA (gDNA) derived from LCM-procured MEE of GD14.5 palate for pyrosequencing. Four of the six genes were amplified by PCR analysis. **A, right panel.** Amplification of *Dkk4* using freshly isolated, bisulfite-modified LCM gDNA. Primers and the region for analysis were determined using the PyroMark Assay Design 2.0 (Qiagen) software. M, 100 bp marker. **B.** Pyrosequencing of *Aph1a* and *Dkk4*. The PCR products from *Aph1a* and *Dkk4* (asterisks) were used for pyrosequencing. Methylation level of the single CpG site analyzed within *Aph1a* (left) was 78% whereas that for the 3 contiguous CpG residues for *Dkk4* (right) were 3%, 97% and 99%. For *Dkk4*, a re-amplified PCR product was used for pyrosequencing. Pyrosequencing was done on a PyroMark Q24 system (Qiagen).

Table 1

Yield and quality of nucleic acids isolated by the LCM protocol

	<u>Number of pulses</u>	<u>LCM caps used</u>	<u>Total yield</u>	<u>Yield/pulse</u>	<u>(N)</u>
RNA:	2058.3 ± 936.8	15.3 ± 3.0	80.8 ± 35.0 ng	40.3 ± 14.2 pg	(6)
DNA:	1322.5 ± 584.8	12.0 ± 4.2	4.9 ± 0.8 µg	4.0 ± 1.2 ng	(2)
RIN:	8.6 ± 0.6 (N = 6)*				

Values represent Mean ± S.D of N samples;

* RNAs from LCM-procured medial edge epithelia isolated from unfixed Gd13.5 tissues.

Table 2

Effect of fixation on RNA quality

<u>Sample</u>	<u>Fixed</u> ¹	<u>Ct ± S.D.</u> ²	<u>(N)</u>
Scrape	No	14.8 ± 0.3	(3)
Scrape	Yes	14.4 ± 0.1	(3)
LCM	No	16.5 ± 0.1	(6)
LCM	Yes	16.4 ± 0.1	(6)

¹ Fixation protocol: ice-cold acetone:75% ethanol (1:1) – 2 min; 75% ethanol - 2 min; 95% ethanol - 15 sec; 100% ethanol – 5 sec; xylene - 4.5 min;

² RNA quality assessed by 18S rRNA Taqman assay (Life Technologies);

Ct, cycle threshold; N, number of samples;

RNAqueous® Micro kit vs Picopure® RNA isolation kit - Expression of miR-99a and miR-200b in tissue scrapes and LCM samples

Table 3

<u>Ct values</u>			
<u>Tissue scrapes</u>	<u>snoRNA-135</u>	<u>miR-99a</u>	<u>miR-200b</u>
RNAqueous	25.52 ± 0.08**	28.12 ± 0.09**	32.47 ± 0.38**
Picopure	27.60 ± 0.10	37.17 ± 1.52	38.24 ± 0.62
<u>LCM isolated</u>			
RNAqueous	28.97 ± 0.03	34.36 ± 0.32	36.51 ± 0.81*
Picopure	28.75 ± 0.14	33.22 ± 0.23**	39.53 ± 0.92
MNP (GD10)	25.01 ± 0.14	27.54 ± 0.20	29.63 ± 0.24

A minimum of 3 independent samples each were used for tissue scrapes and LCM samples; values represent Mean ± S.D. of a representative sample run in triplicate; the mirVana kit (Ambion) was used for the isolation of small/micro RNAs from the medial nasal process (MNP);

* p < 0.05;

** p < 0.01

RNAqueous® Micro kit vs Picopure® RNA isolation kit – Expression of *Tgf-β3* and *Mmp-13* in cell scrapes and LCM samples**Table 4**

<u>Ct values</u>				
<u>Tissue scrapes</u>	<u>18S</u>	<u><i>Mmp-13</i></u>	<u><i>Tgf-β3</i></u>	
RNAqueous	16.52 ± 0.01	28.12 ± 0.09**	32.47 ± 0.38	
Picopure	16.48 ± 0.04	34.09 ± 0.22	32.95 ± 0.29	
<u>LCM isolated</u>				
RNAqueous	20.55 ± 0.03	33.82 ± 0.89**	35.99 ± 0.62	
Picopure	20.37 ± 0.03**	> 40	37.08 ± 0.83	
Whole Palate (GD13.5)	15.73 ± 0.02	> 40	31.94 ± 0.14	

A minimum of 3 independent samples each were used for tissue scrapes and LCM samples; values represent Mean ± S.D. of a representative sample run in triplicate; the RNeasy kit (Qiagen) was used to isolate RNAs from whole palate tissue;

**
p < 0.01