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Comparative performance analyses of commercially available products for salivary collection and nucleic acid processing in the newborn

JL Maron¹ and KL Johnson²

¹Mother Infant Research Institute at Tufts Medical Center, Division of Newborn Medicine, 800 Washington Street, Box 394, Tufts University Boston, Massachusetts

²Tufts University School of Medicine, 800 Washington Street, Box 394, Tufts University Boston, Massachusetts

Abstract

Analysis of saliva for clinical monitoring and biomarker detection holds great promise for improving health care. Commercially available assays are not intended for use with neonates, however, and collection and processing of saliva for subsequent transcriptomic analysis presents unique challenges in this population. We compared RNA yield, quality, stability and RT-qPCR performance for two commonly used commercial systems: the Qiagen RNeasy Protect Saliva Mini Kit® and the DNA Genotek Oragene•RNA® assay. Two 10 µl saliva samples were collected from ten newborns and stabilized for each assay. Total RNA was extracted following incubation for 3, 10, 15 or 20 days. Total RNA extracted from each assay was analyzed for integrity, quality and quantity using the Agilent BioAnalyzer 2100. RT-qPCR was performed for the reference gene, *GAPDH*, to assess subsequent performance of the extracted RNA. Although the DNA Genotek extraction protocol required nearly twice the time of the Qiagen protocol, RNA integrity did not differ between the kits. RNA concentration using the DNA Genotek assay, however, was 3,264 pg/µl (range: 262–10,336 pg/µl) compared to 822.4 pg/µl (range: 0–1,856 pg/µl) for the Qiagen protocol. Linear regression analysis showed a stronger correlation between the threshold cycle and RNA concentration using DNA Genotek ($r^2 = 0.356$) compared to Qiagen ($r^2 = 0.0331$). Our results suggest that although the Qiagen assay may reduce overall extraction time, RNA yield and performance in subsequent transcriptomic analysis is more robust using the DNA Genotek assay.

Keywords

DNA Genotek; newborn; nucleic acids; Qiagen; saliva

Salivary diagnostics is a rapidly emerging field in medicine because saliva can be collected easily and safely. The use of saliva has contributed to new diagnostic panels for infectious disease (Béclec et al. 2011, Parisi et al. 2013), oncology (Brinkmann et al. 2011a) and

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Correspondence: Jill L. Maron M.D., M.P.H., Mother Infant Research Institute, Division of Newborn Medicine, Tufts Medical Center, 800 Washington Street, Box 394, Boston, MA 02111. Phone: 617-636-0766.

Declaration of interest: DNA Genotek donated the modified collection devices and assay kits used in this study. The company did not participate in study design, data analysis or manuscript preparation.

inflammatory processes (e.g., periodontal disease) (Brinkmann et al. 2011b). Commercially available assays for salivary collection and nucleic acid analysis, however, have been designed for adults and older children for whom collection of saliva is voluntary and milliliter amounts can be acquired readily. Salivary volumes are limited to microliters for newborn and premature neonates; therefore, protocols must be optimized for nucleic acid recovery from this unique population. We modified earlier commercially available protocols for optimal RNA recovery from neonatal salivary supernatants (Dietz et al. 2011). Despite the success of this approach, limitations for neonatal salivary processing include technical difficulties for salivary acquisition and optimal nucleic acid stabilization for large scale multicenter studies and studies conducted in developing nations.

We compared commercially available approaches using modified protocols for salivary nucleic acid collection, stabilization and recovery from premature newborns. We compared the Qiagen RNeasy Protect Saliva Mini Kit® protocol (Qiagen, Valencia, CA) to the DNA Genotek Oragene®RNA assay (DNA Genotek Inc., Kanata, Ontario, Canada) for the following parameters: (1) ease of collection, processing and storage, (2) RNA stabilization for increasing intervals of time, (3) RNA yield, and (4) performance on the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) platform. We attempted to assess advantages and disadvantages of both assays for developing neonatal salivary diagnostics.

Material and methods

Our study was approved by the Tufts Medical Center Institutional Review Board. Informed parental consent was obtained for each subject enrolled. Ten neonatal subjects participated in our study; pertinent demographic and clinical data for each subject are given in Table 1.

Collection of saliva

Samples of saliva were collected using previously established protocols from our laboratory (Maron et al. 2010). Briefly, a 1 ml syringe with end caps removed was connected to wall vacuum (−40 mm Hg). The subject's oropharynx was suctioned for less than 1 min, specifically targeting gingival crevices and under the tongue where saliva is known to pool. Saliva samples generally were collected 1 h after feeding to avoid contamination by breast milk or formula. Two 10 µl samples were collected sequentially for processing with each kit. To avoid bias, the order of sample collection was alternated for use with each kit.

Stabilization of saliva

Qiagen RNeasy Protect Saliva Mini Kit®—To stabilize saliva for use with the Qiagen RNeasy Protect Saliva Kit®, 500 µl of RNAprotect Saliva Reagent® was aliquoted into 2 ml Eppendorf tubes. RNAprotect Saliva Reagent® is supplied with the Qiagen RNeasy Protect Saliva Mini Kit® and is designed to halt gene expression changes, limit microbial overgrowth and inhibit destructive RNases. The manufacturer's directions suggest that 200 µl of saliva be placed directly in 1 ml of the RNAprotect Saliva Reagent®; however, we modified this protocol for use in newborns. Approximately 10 µl of saliva was placed in 500 µl of RNAprotect Saliva Reagent® by flushing the collection syringe with the reagent 4–5

times to ensure that all saliva was removed from the syringe. The Eppendorf tubes then were vortexed, placed immediately on ice and stored at 4° C until total RNA extraction.

DNA Genotek Oragene•RNA® assay—A modified collection system has been developed by DNA Genotek to stabilize salivary RNA for use with the DNA Genotek Oragene•RNA Kit® for newborns. The new procedure for newborns is similar to the Oragene•Discover system (formerly Oragene•RNA), which calls for the subject to provide 2 ml of saliva into a collection vessel attached to a flip cap that contains the stabilizing agent aimed at halting gene expression changes, inhibiting microbial growth and destroying ubiquitous RNases. After collection of saliva, the subject closes the cap, which breaks a seal to release 2 ml of stabilizing agent directly onto the saliva sample. The solutions then are mixed thoroughly and may be stored at room temperature for up to eight weeks. We modified the device to contain 1 ml of stabilizing agent within the sealed, breakable cap. In our study, the seal was broken immediately prior to neonatal saliva collection. Once the sample was collected, approximately 10 µl of saliva was flushed with the stabilizing solution using the collection syringe to remove and stabilize the saliva sample. The solutions then were mixed and stored at room temperature until total RNA extraction.

Incubation periods

Samples were stored at either 4° C (QiagenRNeasy Protect Saliva Mini Kit®) or room temperature (DNA Genotek Oragene•RNA® assay). Incubation periods were 3 days (subjects 1 and 2), 10 days (subjects 3–5), 15 days (subjects 6–8) or 20 days (samples 9 and 10).

Extraction of salivary RNA

Qiagen RNeasy Protect Saliva Mini Kit®—Total RNA extraction using the Qiagen RNeasy Protect Saliva Mini Kit® was performed according to the manufacturer's instructions. The kit uses a silica-membrane RNeasy spin column capable of binding up to 100 µg of total RNA. Each sample underwent treatment with DNase to destroy any residual genomic DNA that would interfere with RNA amplification on the RT-qPCR platform with the use of Qiagen's RNase-free DNase Set according to the manufacturer's instructions. Fresh 70 and 80% ethanol solutions were used during each extraction procedure. The final elution volume of extracted total RNA was approximately 12 µl. Samples were stored at –80° C until further analysis.

DNA Genotek Oragene•RNA® assay—Total RNA extraction using the DNA Genotek Oragene•RNA® assay was performed according to manufacturer's instructions. DNase treatment was performed on all samples to eliminate genomic contamination using Qiagen's RNase-free DNase Set according to the manufacturer's instructions. Fresh 80 and 95% ethanol solutions were prepared on each day of extraction. The final elution volume was approximately 22 µl. Samples were stored at –80° C until further analysis.

Qualitative assessment of salivary RNA

All 20 extracted samples from both protocols were analyzed using the Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Santa Clara, CA) according to the manufacturer's

instructions. Total RNA quantity and quality were assessed as the RNA integrity number (RIN). The RIN was determined based upon the algorithm developed by Agilent Technologies that uses electrophoretic separation of total RNA in each sample to generate a ribosomal ratio (Mueller et al. 2004). RINs range from a score of 1 (most degraded) to 10 (most intact)

Transcriptomic applications: reverse transcription quantitative-polymerase chain reaction (RT-qPCR)

To assess the performance of extracted mRNA, we performed RT-qPCR for the reference gene, *GAPDH*. We have shown earlier that *GAPDH* is a stable reference gene among premature neonates (Maron et al. 2012). Owing to limited sample volume, each sample was run only once. All samples were analyzed using the Applied Biosystems® 7900 with TaqMan One-Step Master Mix® and reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Primer and probe sequences for *GAPDH* were: *GAPDH* forward, 5'-GAAGGTGAAGGTCGGAGTC-3', *GAPDH* reverse, 5'-GAAGATGGTGATGGGATTTC-3', *GAPDH* probe, 5'-(6-FAM)-CAAGCTTCCCGTTCTCAGCC-(TAMRA)-3'. Negative controls with nuclease-free water were run on the plate. The total volume for each reaction was 50 µl including 5 µl of sample in each well. The thermal cycle profile was: 48° C for 10 min, 95° C for 10 min, followed by 40 cycles of PCR with a 15 sec denaturing cycle at 95° C followed by 45 sec annealing and extension at 60° C.

Statistical analysis

Paired two-sided student t-tests were performed for comparison of the assays for the following outcomes: RINs, total RNA quantity and threshold cycle (Ct) on RT-qPCR. A one-sided ANOVA was performed to assess RNA quantitative yield and integrity differences as determined by the BioAnalyzer and RT-qPCR experiments with increasing incubation times. Linear regression analyses were performed to assess the relation between quantitative total RNA and Ct on RT-qPCR for *GAPDH* for each assay. A value for $p < 0.05$ was considered significant.

Results

Collection and storage of saliva

Salivary collection was comparable for both protocols. DNA Genotek, however, has a pre-manufactured system that eliminates the need to pre-aliquot stabilizing agents. In addition, the DNA Genotek Oragene•RNA® system is stable at room temperature for up to 8 weeks, while the Qiagen RNA Protect Saliva Mini Kit® has more limited storage options. Saliva stabilized with the Qiagen RNA Protect Saliva Mini Kit may be stored at room temperature for up to 14 days, with the option to store samples at 2–8° C for up to 4 weeks, or at –20 or –80° C for extended periods.

RNA extraction

Both the Qiagen RNeasyProtect Saliva Mini Kit® and DNA Genotek Oragene•RNA® assay incorporate Qiagen's on-column RNA extraction products and protocol. The DNA Genotek

Oragene•RNA® system, however, requires several extended incubation periods (50° C × 1 h, 90° C × 15 min, 4° C × 10 min and -20° C × 30 min), which nearly doubles the time required for RNA extraction.

RNA integrity

RNA quality was the same for both kits. The average RIN for the DNA Genotek Oragene•RNA® assay was 2.28 (range: 1.2–2.7), while the average RNA for the Qiagen RNeasy Protect Saliva Mini Kit® was 2.56 (range: 0–6.1) ($p = 0.53$).

RNA quantity

There was a statistically significant difference in total RNA yield between the two methods produced. The mean concentration of total RNA for the DNA Genotek Oragene•RNA® assay was 3264 pg/μl (range: 262–10336 pg/μl) compared to 822.4 pg/μl (range: 0–1856 pg/μl) for the Qiagen RNeasy Protect Saliva Mini Kit® ($p = 0.02$).

RT-qPCR performance

Despite a significantly greater amount of total RNA produced by the DNA Genotek Oragene•RNA® samples, differences in mean Ct for the reference gene, *GAPDH*, between groups were not statistically significant. The mean Ct for DNA Genotek and Qiagen RNeasy Protect Saliva Mini Kit® were 29.03 (range: 24.88–34) and 29.7 (range: 26.64–33), respectively ($p = 0.6$).

Incubation effect

Increasing incubation times produced no difference in either RNA yield or integrity. RNA concentrations determined by the BioAnalyzer were not significantly different for the two kits (ANOVA p values: DNA Genotek $p = 0.3$; Qiagen $p = 0.12$). Similarly, Ct values were similar with either kit (ANOVA p values: DNA Genotek $p = 0.9$; Qiagen $p = 0.78$). Finally, the integrity of RNA with increasing incubation times was not significantly different (ANOVA p values: DNA Genotek $p = 0.73$; Qiagen $p = 0.37$).

Linear regression analysis

The relation between RNA concentration and Ct was stronger with DNA Genotek Oragene•RNA® ($R^2 = 0.356$) (Fig. 1a) than for the corresponding assessment for the Qiagen RNeasy Protect Saliva Mini Kit® ($R^2 = 0.03$) (Fig. 1b).

Discussion

The acquisition and subsequent analysis of neonatal salivary samples presents unique challenges. Whether born prematurely or critically ill at term, infants in neonatal intensive care units can produce only small amounts of saliva compared to older populations for which commercially available salivary analysis products have been developed. The need to use emerging technology for biomarker discovery and improved noninvasive monitoring of neonates is great (Romano-Keeler et al. 2014). We have found that small modifications to existing protocols and products produce successful results. Our findings can be applied to

any clinical situation where low salivary volumes may prohibit subsequent transcriptomic analysis.

There are benefits and limitations to both kits that we investigated. The user-friendly approach to salivary stabilization offered by DNA Genotek Oragene•RNA® makes it ideal for multicenter or international studies. Each premade kit is opened at the time of salivary collection, which eliminates the need for pre-aliquoting the stabilizing solution. Further, RNA derived from saliva collected with the DNA Genotek Oragene•RNA® system is stable at room temperature for up to 8 weeks, which reduces the need for refrigeration or frequent shipping to central sites compared to the Qiagen® RNeasy Protect Saliva Mini Kit®. Extraction protocols for the two assays varied considerably, however, and the more time-consuming DNA Genotek Oragene•RNA® assay may limit its applicability, particularly for high-throughput situations.

We found that the DNA Genotek Oragene•RNA® system yielded significantly more total RNA than the Qiagen RNeasy Protect Saliva Mini Kit® despite comparable overall Ct values as determined by RT-qPCR for the reference gene, *GAPDH*, for the two assays. The BioAnalyzer 2100 measures total RNA concentration, however, which is predominantly ribosomal, while RT-qPCR quantifies mRNA exclusively. Therefore, differences in Ct values and RNA quantity likely reflect the relative abundance of different RNA species measured by each technique.

Linear regression analysis that demonstrated the relation between RNA concentration and Ct revealed a more robust relation using the DNA Genotek Oragene•RNA® system. Ct levels decreased steadily with increasing amounts of RNA that were stabilized and extracted using the DNA Genotek Oragene•RNA® system. Conversely, the Qiagen RNeasy Protect Saliva Mini Kit® showed no such relation. These data suggest that more overall RNA was extracted using the DNA Genotek Oragene•RNA® system and that it may perform better on the RT-qPCR platform. Future studies are required to assess performance differences using additional transcriptomic platforms including gene expression microarray analyses and RNA sequencing.

Despite the quantitative differences produced by the two extraction protocols, there was no difference in quality of total RNA extracted with either kit. Overall, extracted total salivary RNA was of poor quality (RIN < 7.0) for both assays. These findings were expected, because salivary RNA is derived from a mixed source of cellular and cell-free elements. Incubation intervals did not affect these results. All samples performed well for subsequent RT-qPCR.

A limitation of our study was our inability to assess RNA quantity and quality of the same sample at different time intervals. We investigated quantitative and qualitative differences in RNA from different samples over time. Both DNA Genotek and Qiagen have reported the ability of their stabilizing agents to prevent RNA degradation over time and we did not repeat those experiments. In addition, our results were derived from a limited cohort of ten subjects; it may not be possible to generalize our observations to a wide spectrum of infants at different gestational ages.

Despite the limitations, commercially available salivary nucleic acid systems can be modified and applied successfully to the neonatal population. The DNA Genotek Oragene•RNA® system offers easier collection and stabilization of salivary RNA, which makes it ideal for multicenter use. Further, this system yielded a higher concentration of salivary RNA, which was better correlated to performance by RT-qPCR compared to the Qiagen RNeasy Protect Saliva Mini Kit®. The Qiagen extraction protocol is more user-friendly, however, particularly for large clinical trials when high throughput is essential. Investigators should consider study design and ease of processing when choosing a product and not be deterred by the unique challenges of the neonatal population.

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A:

b:

Fig. 1.

A) Linear regression analysis of Ct vs. RNA concentration using the DNA Genotek Oragene•RNA® system. B) Linear regression analysis of Ct vs. RNA concentration using the Qiagen RNeasy Protect Saliva Mini Kit®.

Table 1

Subject demographics

Subject	Gender	Post-conception age (weeks)	Weight (kg)
1	Male	30 3/7	1.377
2	Male	35	2.317
3	Male	32 6/7	1.365
4	Female	32 6/7	1.625
5	Male	41 4/7	3.795
6	Male	35	1.977
7	Male	36 2/7	1.838
8	Female	34	2.136
9	Male	35 1/7	2.409
10	Male	35 1/7	2.352

Table 2

BioAnalyzer results

Subject	DNA Genotek RIN*	DNA Genotek concentration (pg/μl)	Qiagen RIN*	Qiagen concentration (pg/μl)
1	2.3	6,395	N/A	0
2	2.7	2,061	2.5	1,582
3	2.5	306	2.3	276
4	2.4	3,008	6.1	563
5	1.2	262	2.4	514
6	2.7	3,982	1	336
7	2.2	2,720	1.7	707
8	2	10,336	2.4	791
9	2.5	2,281	2.4	1,856
10	2.3	1,284	2.4	1,599

* RNA integrity number

Table 3

RT-qPCR results

Subject	DNA Genotek (Ct [*])	Qiagen® (Ct [*])
1	24.88	27.66
2	34	33
3	31.4	31.4
4	25.68	26.64
5	31.46	27.4
6	29.72	32.26
7	27	30
8	27.04	29.65
9	28.74	27.1
10	30.71	31.86

* Threshold cycle