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# Clinical trials optimizing investigator and self-collection of buccal cells for RNA yield

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## Abstract

**Objective:** Buccal cells are an ideal surrogate tissue for studying biologic effects of carcinogens or drugs, however inherent fragility and salivary RNases limit RNA yield. We conducted healthy volunteer trials to optimize collection conditions.

**Methods:** We conducted: (a) a single-arm crossover study evaluating four test conditions on RNA yield by buccal cytobrush; (b) a single-arm prospective study evaluating RNA yield by investigator vs self-collection.

**Results:** Antecedent toothbrushing, time of day, and number of cytobrush strokes did not significantly impact RNA yield. RNA yield was doubled by using 2 vs 1 cytobrush per buccal surface ( $P = .0054$ ). Self-collection of buccal cells for RNA was feasible; 36 of 50 (72%) samples passed quality control.

**Conclusion:** RNA yield was doubled by using two cytobrushes per buccal surface. Healthy volunteers can self-collect sufficient buccal RNA for gene expression studies. Techniques from these pragmatic trials could enhance availability of a limited tissue for serial biomarker measurements.

**Level of Evidence:** 1b—Prognosis Study (Individual prospective cohort study).

## KEYWORDS

biomarker, buccal cell, cytobrush, evidence-based medicine, molecular biology, oral cavity, RNA, self-collection

## 1 | INTRODUCTION

Epithelial cancers of the upper aerodigestive tract (UADT), including the mucosal linings of the oral cavity, pharynx, larynx, esophagus, and lung,

share the dominant environmental risk factor of tobacco exposure. Chronic exposure to combustible tobacco results in molecular and histologic changes within grossly normal mucosa. Such changes, first described by Slaughter and colleagues in 1953, led to the concept of “condemned epithelium,” or diffuse premalignant changes occurring within the UADT mucosal field exposed to a common carcinogen.<sup>1</sup> Because most UADT

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cancers do not arise from visually apparent lesions, such as oral leukoplakia, research into non-invasive sampling of the UADT to identify biomarkers of risk, even prior to the development of clinical disease, is ongoing.<sup>2</sup> In particular, buccal cells, epithelial cells that line the inner cheek, have emerged as a promising surrogate tissue for studying the biologic effects of a carcinogen, the presence of preclinical disease, and pharmacodynamic effects of candidate chemoprevention drugs.<sup>3-8</sup> Buccal cells are a particularly attractive biomarker source as they may be obtained non-invasively by cytobrush or mouthwash.

Cost-effective, reliable methods for collecting DNA from buccal cells have been optimized for large scale epidemiologic studies.<sup>9-11</sup> Due to the stability of genomic DNA, even mail-in, self-collection by oral cytobrush or mouthwash is sufficiently robust to conduct genome and epigenome-wide association studies on buccal DNA samples many years later.<sup>11,12</sup> However, buccal RNA expression is of much greater utility in studying dynamic, mechanistic changes within at-risk tissue during carcinogen exposure or pharmacologic intervention. For example, quantitative analysis of buccal gene expression has demonstrated differential expression of genes related to oxidative stress, oncogenesis, tumor suppression, and regulation of inflammation in smokers vs non-smokers.<sup>3,4,7</sup> However, the collection of RNA from buccal cells has inherent challenges that limit RNA yield. First, RNA is known for its rapid degradation, with half-lives as brief as 12 minutes necessitating rapid processing.<sup>13</sup> Moreover, unlike genomic DNA, RNA can only be harvested from viable cells, which comprise only 5% to 23% of non-invasively collected buccal cells, and thus limits quantity of RNA per collection.<sup>14</sup> Second, oral saliva contains RNases that facilitate the first phase of digestion, which compromise both the quantity and quality of collected buccal RNA.<sup>15</sup> Although immediately plunging the buccal specimen into an RNA preservative markedly enhances yield, the need for specialized training, collection tools, and processing materials has reduced the feasibility of both investigator collection at multiple time points and of self-collection, which has never been studied.

To facilitate the use of serial, non-invasive buccal cell sampling for pharmacodynamic endpoints in clinical trials, in particular UADT cancer chemoprevention trials, we performed iterative, pragmatic collection experiments in healthy volunteers to establish the optimal conditions for maximal RNA yield. Although multiple test conditions including toothbrushing before collection, brush strokes for cytobrush, time of day, and self-collection techniques have been evaluated in protocols optimizing buccal DNA collection,<sup>9-11</sup> these variables have not been studied in buccal RNA collection. In addition, we conducted a prospective trial evaluating the feasibility of self-collection. If feasible, self-collection would permit serial analysis of buccal RNA for pharmacodynamic gene expression studies, greatly enhancing mechanistic knowledge gained during clinical trials.

## 2 | METHODS

### 2.1 | Clinical trial design

UCSF 16-20427 was approved by the Institutional Review Board of the University of California San Francisco and all subjects provided

written, informed consent. This was a prospective, single-arm cross-over study in healthy adult volunteers evaluating the effect of four specific test conditions on the RNA yield obtained by buccal cytobrush performed by the same trained investigator (EC). Subjects served as their own controls. Specific collection methods were sequentially varied in four sub-experiments as follows: (a) collection before and after toothbrushing; (b) number of cytobrush strokes (10 vs 20); (c) time of day (6-8 AM vs 3-5 PM); (d) number of cytobrushes used per collection (1 vs 2). In each sub-experiment, 10 subjects underwent an initial baseline collection from one buccal surface, and a second collection from the opposite side after changing a single variable. The collection method yielding the greater concentration of RNA served as the baseline collection condition (control) for the following sub-experiment. In the absence of a statistically significant difference in RNA yield, the most convenient method was used as the baseline for the next sub-experiment. All sub-experiments were conducted with a minimum 1-week interval, to allow for regeneration of oral mucosa. The following variables were kept constant across all sub-experiments: All collections were performed at least 2 hours after a meal and after rinsing the mouth with water (10-second swishes) three times. Cytobrushes were plunged immediately into vials containing 1.5 mL of RNA<sup>later</sup> solution (Life Technologies) and stored on ice until being frozen at  $-80^{\circ}\text{C}$  within 6 hours of collection. For each sub-experiment, the difference in within-subject RNA yield was evaluated by two-tailed Wilcoxon signed-rank test, with alpha set at 0.05.

UPCI 15-204 was approved by the Institutional Review Board of the University of Pittsburgh and registered at clinicaltrials.gov (NCT02800265). Part 1, a prospective cohort study evaluating the feasibility of buccal cell self-collection by healthy adult volunteers, is reported here. Part 2, a subsequent prospective cohort study in the same subjects evaluating modulation of buccal cell gene expression by a putative cancer prevention agent, is ongoing. All subjects provided written, informed consent. Eligible subjects were healthy individuals aged  $\geq 18$  years willing and able to perform self-collection of buccal cells. Participants received a \$50 gift card upon completion of study requirements. During part 1 of the study, each healthy subject underwent buccal cell collection by a single cytobrush once daily for 5 days. On days 1 and 2, the trained investigator (JG) collected buccal cells for RNA from the right buccal mucosa, while demonstrating the appropriate technique. This technique included using the ipsilateral hand to better expose the buccal mucosa, while inserting the cytobrush into the oral cavity and directing toward the cheek wall. Light pressure was applied against the buccal mucosa, and the cytobrush was then swabbed in the same direction with 5 to 10 vigorous strokes. The brush was then immediately placed in the tube of RNA<sup>later</sup> and swirled and tapped in the tube to transfer the swabbed cells into the solution. The investigator then observed each subject perform self-collection from the left buccal mucosa. Each subject was provided a 3-day buccal cell collection kit and written instructions. On days 3 to 5, the investigator collected daily buccal mucosa samples on the right side in clinic, while subjects collected daily samples from the left side at home. All samples were immediately transferred into

individual cryovials containing 1.5 mL of RNAlater. Samples collected in clinic were stored at  $-80^{\circ}\text{C}$  until analysis. Self-collected samples were stored in the subject's home freezer (approximately  $-20^{\circ}\text{C}$ ) until they were returned on ice packs to clinic on day 5, when they were transferred to the  $-80^{\circ}\text{C}$  freezer.

The feasibility of buccal cell collection for RNA was determined by the proportion of time points where the yield was at least 20 ng/ $\mu\text{L}$  (corresponding to an absolute quantity of 500  $\mu\text{g}$  of RNA), the minimum concentration required for quantitative PCR using pre-defined quality control (QC) criteria.<sup>5</sup> Investigator collections were judged feasible if  $>80\%$  of time points met minimum yield, and self-collection was deemed feasible if  $>60\%$  of time points met minimum yield. Mixed effects ANOVA was applied to characterize the differences in RNA yield between the investigator and self-collection routines, where the fixed effects were the two collection methods and the random effect was trial participant. The null hypothesis was tested by mixed effects logistic regression.

## 2.2 | RNA purification, quantification, and quality analysis

Buccal cell samples were stored in cryovial tubes containing RNAlater at  $-80^{\circ}\text{C}$  until laboratory analysis (DJ). For UCSF 16-20 427, total RNA was purified from human buccal cells using the miRNeasy Mini Kit (Qiagen). For UPCI 15-204, total RNA was purified from human buccal cells using the RNAqueous-Micro Total RNA Isolation Kit (Thermo Fisher Scientific). RNA concentration was measured by the NanoDrop spectrophotometer (Thermo Fisher Scientific) as well as Qubit fluorometric quantification. The quality of RNA from UPCI 15-204 samples was examined using Fragment Analyzer assessment.<sup>16</sup>

## 3 | RESULTS

### 3.1 | RNA yield is significantly improved by the use of two cytobrushes per collection, and is not affected by antecedent toothbrushing, time of day, or number of cytobrush strokes

From November to December, 2016, 19 healthy volunteers aged 25 to 65 were accrued to UCSF 16-20427. Ten subjects were included in each sub-experiment 1 to 4.

RNA yields from each sub-experiment are shown in Table 1. As toothbrushing prior to mouthwash collection of buccal cells has been found to reduce DNA yield by 40%, this condition was tested in sub-experiment 1.<sup>9</sup> There was no significant difference in RNA yield for buccal collection by cytobrush, before vs after toothbrushing (median 35.6 vs 30.2 ng/ $\mu\text{L}$ ;  $P = .74$ ). Therefore, all subsequent sub-experiments were performed after toothbrushing, due to subject convenience. Mouthwash swish time and number of cytobrush strokes have been evaluated and found to have no impact on buccal cell DNA yield.<sup>9,10</sup> Similarly, in sub-experiment 2, no statistical difference was observed for 10 vs 20 cytobrush strokes (median 19.6 vs 19.0 ng/ $\mu\text{L}$ ;  $P = .35$ ). Thus, 10 strokes were used for the remaining sub-experiments. Diurnal variation in metabolic activity of buccal cells, with a morning peak, may affect buccal cell yield and the consequent harvest of genetic material. Although buccal cell DNA yield was not improved by restricting collection to the morning only,<sup>10</sup> RNA yield comes only from viable cells and may be more sensitive to metabolic fluctuations. However, in sub-experiment 3 no significant difference in RNA yield was observed for morning vs afternoon collection (median 8.8 vs 11.0 ng/ $\mu\text{L}$ ;  $P = .59$ ). Thus, morning collection was used for the subsequent sub-experiments. Notably, using 2 vs 1 cytobrush (10 strokes each) per collection significantly increased RNA yield, nearly doubling

**TABLE 1** mRNA yields by toothbrushing, cytobrush strokes, time of collection, and number of cytobrushes

Subject number	Sub-experiments 1–4 (mRNA concentration in ng/ $\mu\text{L}$ )							
	1		2		3		4	
	Before tooth-brushing	After tooth-brushing	20 Strokes	10 Strokes	AM	PM	2 Cytobrush	1 Cytobrush
1	41.08	47.88	30.68	37.56	23.8	16.92	28.96	16.48
2	23.32	32.52	117.64	93.8	10.2	13.12	27.36	11.08
3	44.76	27.88	30.44	36.6	14.92	8.72	25	16.8
4	17.56	19	11.56	4.12	14.76	5.16	39.92	34
5	38.96	67.4	15.8	14.04	24.12	36.08	44.32	21
6	32.76	183.2	21.56	24.76	7.32	3.84	29.24	8.4
7	101.32	93.04	126.36	26.04	6.96	22.8	16.72	6.36
8	14.52	21.16	14.12	11.24	2.32	3.04	45.2	16.44
9	38.52	12.12	10.84	14.36	5.08	15.4	51.72	22.84
10	28.44	20.68	16.36	8.2	7.32	7.92	51	26
Average mRNA	38.12	52.49	39.54	27.07	11.68	13.30	35.94	17.94
Median mRNA	35.64	30.2	18.96	19.56	8.76	10.92	34.58	16.64
Wilcoxon 2-tail	$P = .7414$		$P = .3472$		$P = .5892$		$p = .0054$	

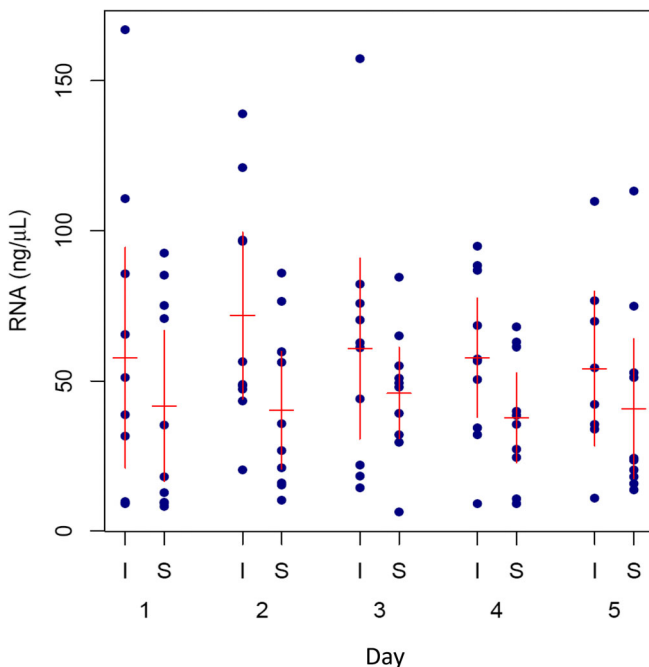
RNA concentration (median 34.6 vs 16.6 ng/ $\mu$ L;  $P = .0054$ ). As increasing the number of strokes per cytobrush did not increase RNA yield, this suggests that the surface area of each cytobrush becomes saturated after 10 strokes. Collecting additional available buccal cells at the same setting requires a fresh cytobrush.

### 3.2 | Self-collection of buccal cells for RNA is feasible

From May to June 2016, 10 healthy subjects aged 28 to 55 were accrued to UPCI 15-204. The individual collection data by time point is shown in Figure 1 and in Table S1. Two participants were not present for Investigator collection on Day 5; all self-collection samples were accounted for.

In our previous healthy volunteer study, buccal cell collection with a curette by a trained investigator resulted in inadequate RNA yield for quantitative RT-PCR (qPCR) at 20 of 120 (17% of time points)<sup>5</sup>; a similar proportion of low buccal RNA producers has been observed by other laboratories, which has been compensated by pooling multiple time points per subject or adding an amplification step prior to qPCR.<sup>3,5,7</sup> In the current study, we were interested in the feasibility of self-collection of sufficient buccal RNA by cytobrush for qPCR at a single time point, without an amplification step, to justify the use of inexpensive, serial buccal cell sampling for

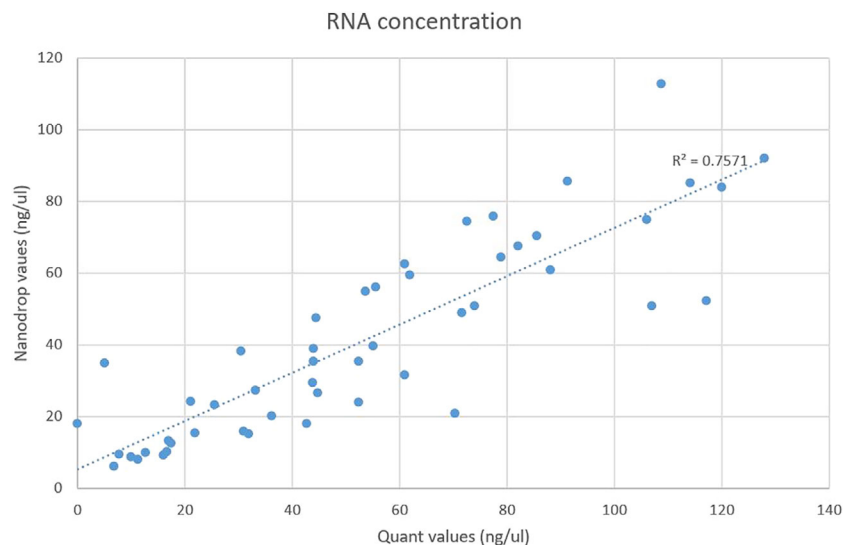
pharmacodynamic analyses in future chemoprevention studies. Collection by a trained investigator results in acceptable yield approximately 80% of the time, thus self-collection was defined as feasible if subjects obtained the minimal acceptable RNA yield 60% of the time. In this study, investigator collection yielded 9 to 166.9 ng/ $\mu$ L of RNA per sample (median 54.2 ng/ $\mu$ L; mean 57.7 ng/ $\mu$ L; SD = 51.3), and 41 of 48 (85%) of investigator-collected samples were above the minimal acceptable yield of RNA, in line with expectations. Self-collection yielded 8.2 to 113.1 ng/ $\mu$ L of RNA per sample (median 35.5 ng/ $\mu$ L; mean 41.6 ng/ $\mu$ L; SD = 35.1), with 36 of 50 (72%) samples meeting QC criteria for acceptable yield. Baseline yields were right-skewed and the SD was proportional to the mean, indicative of high variance. However, variance in RNA yield was not of interest as an analysis variable. The primary endpoint was the practical assessment of whether sufficient RNA was present for downstream molecular analysis, that is, did the total quantity exceed a predetermined threshold concentration. Therefore, per pre-specified study design for the primary study endpoint, both investigator- and self-collection by cytobrush were judged feasible for obtaining adequate buccal cell RNA for gene expression studies by quantitative RT-PCR. Per mixed effects ANOVA, there was no significant difference across days for either investigator or self-collection method. Nonetheless, only 5 of 10 participants (50%) collected sufficient samples on Day 1, which was lower than any other day (70%-90%), likely reflecting a brief learning curve. As displayed in Figure 1, investigators collected significantly more RNA than did subjects ( $P = .0006$ ), however both methods were feasible.



**FIGURE 1** Investigators collected significantly more mRNA across all days than did subjects, however both collection methods were feasible. Data are displayed by day on the x axis (“I” = Investigator and “S” = Self), with each point representing the mRNA concentration of a single collection. The horizontal hatch mark represents the mean investigator or self-collection for that day, and the intersecting vertical line represents the 95% confidence interval

### 3.3 | RNA quality analysis

To confirm the quantity of RNA extracted from self-collected buccal cell specimens, the samples from UPCI 15-204 were further examined using Qubit fluorometric quantification. As shown in Figure 2, the correlation between the two RNA quantification methods was high (Pearson correlation coefficient,  $R^2 = 0.76$ ). The quality of RNA within investigator and self-collected buccal specimens was subsequently evaluated by Fragment Analyzer (Agilent). All samples were run using an internal control for standardization. Fragment analysis showed that genomic DNA was present in the samples. The RNA from all samples, including investigator and self-collection, showed a degree of degradation based on the detection of peaks of varying sizes ranging between 200 and 600 nucleotides in length, indicating the presence of small nucleotide length RNA (Figure S1). The presence of genomic DNA (human or bacterial) and the fragmentation of RNA are frequently observed within buccal specimens, and do not preclude analysis by common molecular applications including RT-PCR and the NanoString Technologies nCounter platform, which include preparation steps to mitigate common quality issues such as the presence of genomic DNA<sup>17</sup> Both of these molecular applications are planned in Part 2 of UPCI 15-204 and the specimens have passed quality control to proceed with these analyses.



**FIGURE 2** RNA concentration from self-collected buccal cytobrush scrapings were evaluated by both Nanodrop (y-axis) and Qubit fluorometric quantification (x-axis). Correlation between the two assays was high as assessed by Pearson product moment correlation ( $R^2 = 0.76$ )

## 4 | DISCUSSION

Buccal cells, epithelial cells that line the inner cheek, are an ideal surrogate tissue for pharmacodynamic assays in clinical trials for prevention or treatment of epithelial malignancies, in particular UADT cancers, for multiple reasons. As epithelial cells, buccal cells represent a more biologically relevant surrogate than peripheral blood mononuclear cells (PBMCs), the living tissue of convenience used most frequently in therapeutic clinical trials. In the specific case of UADT cancers, buccal cells are representative of the condemned epithelium. Viable buccal cells may be obtained non-invasively by oral brushings, scrapings, or rinses then deployed for pharmacodynamic assays including gene expression.<sup>3-8,18-21</sup> Sampling of the buccal mucosa is an alternative to collection of PBMCs that is less invasive, presents less discomfort, and requires less specialized training than phlebotomy. In conducting these optimization clinical trials, our primary aims were to maximize RNA yield obtained at a single collection time point by a trained investigator, as well as to determine the feasibility of self-collection. As shown, a maximal yield of RNA can be obtained from a single collection by a trained investigator using two cytobrushes, with 85% of samples meeting QC criteria for gene expression studies. Moreover, self-collection of buccal cells for RNA is feasible with minimal instruction, provision of a home collection kit including cytobrushes and cryovials containing RNA preservative, and temporary storage in the subject's home freezer, with 72% of samples meeting QC criteria by NanoDrop spectrophotometry and verified by Qubit fluorometric quantification. Given both investigator- and self-collection were performed with a single cytobrush in this study, instructions to perform collection with two brushes may further optimize yield. Although the investigator and self-collected samples demonstrated both the presence of genomic DNA and RNA fragmentation, these features are common in RNA isolation and do not preclude analysis by most robust, downstream molecular techniques including RT-PCR and the NanoString Technologies nCounter platform due to built-in sample preparation steps and the specificity of the techniques.<sup>17</sup> Both molecular applications are planned in Part 2 of UPCI

15-204 (NCT02800265) as well as other ongoing chemoprevention studies utilizing serial buccal cell cytobrush collection (NCT03182959, NCT03402230).

Multiple studies have examined methods of collection of buccal cells, although few have identified strategies that specifically preserve RNA, which degrades quickly at room temperature.<sup>13,15</sup> In designing these studies, we identified several variables that were considered to potentially impact the yield of viable cells from buccal sampling. Previous studies optimizing DNA yield have shown that toothbrushing prior to sample collection reduced yield in a mouthwash protocol, that no difference in yield was found for morning compared to anytime cytobrushings, and that repeat cytobrushings from the same cheek increased yield.<sup>9,10</sup> Our results show that toothbrushing, time of day, and doubling the number of strokes with a single cytobrush did not significantly impact RNA yield. However, RNA yield was doubled by using a two cytobrushes on the same buccal surface and depositing material from both into a pooled cryovial. This simple, cost-effective modification of the collection technique essentially doubled the RNA available for analysis.

This study does have an important limitation. The serial sub-experiments were pragmatic and were not powered to account for the high intra-individual variance observed. While the design attempted to control for variance using a single, trained investigator and batch analysis, nonetheless variance in RNA yield was high. Consequently, type 2 error may be present for the conditions of toothbrushing, cytobrush strokes, and time of day. Nonetheless, the application of techniques from these pragmatic clinical studies will enhance the availability of a limited and precious tissue resource, by investigator or self-collection. Although our specific purpose in defining these techniques, including self-collection, is the serial evaluation of gene expression changes in the condemned UADT mucosa during chemoprevention trials in smokers, these collection techniques have broader applicability. Optimal buccal cell collection raises the promise of obtaining serial biomarker measurements for granular pharmacodynamic insights on the biologic effects of carcinogen exposure, risk modification interventions, or pharmacotherapies.



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## CONFLICT OF INTEREST

Authors report no conflict of interest for the presented work.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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