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A Necessary Adjustment of Protocol for Use of DPC Coat-a-Count Total Testosterone Assay with Saliva

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Introduction

Salivary testosterone (T) assessment has been used in varied medical (1) and psychological testing (2) contexts. As with other analytes, this is most efficiently done using a direct assay format with an immobilized, highly specific antibody. Many studies have chosen DPC's coated tube assay for total T (Coat-a-Count Total Testosterone, Diagnostic Products Corp., Los Angeles, CA, USA) and their 1986 protocol for saliva to meet these needs. That protocol makes two changes in the serum diagnostic protocol. First, instead of a 50 μL sample of undiluted serum standards or controls, 200 μL is used for raw saliva, or serum standards and controls that are diluted 20-fold in water. Second, the antibody binding period is extended from 3 h at 37°C to 16–24 h at room temperature. Both serum and saliva protocols add 1.0 mL of ^{125}I-T tracer solution so final assay volumes are 1.05 mL and 1.2 mL, respectively. Stated sensitivity (limit of detection) of the DPC kit is ~4 ng/dL (0.14 nM, 40 pg/mL) or 2 pg/tube for serum with an analytical range of 4–1,600 ng/dL (2–800 pg/assay tube). No alteration of assay sensitivity is noted in the DPC salivary protocol but the analytical range is shifted to 1–80 ng/dL (2–160 pg/assay tube). During validation checks of this assay using DPC's saliva protocol, the kit reagents were unable to reliably measure human salivary total T. Therefore, we now question the validity of results in studies using this kit with DPC's saliva protocol without initial validation and modification. Besides this caution, we offer a simple solution to rectify the problem and to generate reliable results from saliva using this assay kit.

We used DPC's coated tube assay for total T and their protocol for saliva in the context of a psychological evaluation of power motivation in college-age young men (Human Use Clearance on 4/2/97 by IRB M1084-01, Harvard University). Published values of salivary T have been reviewed by Read (3). Generally, adult males average 150–500 pM (43–144 pg/mL) including results from studies using a "gold standard" reference method, gas chromatography/mass spectrometry (4). The highest T is anticipated among young adult males such as those under study (3,5). Adult females averaged < 50 pM (< 14 pg/mL) in the same studies noted by Read (3) and Gould *et al.* (4). Given these values, 200 μL of adult male saliva should contain ~8–29 pg of T while female saliva should contain < 3 pg. It is unlikely the DPC kit and protocol will generate reliable results for female samples as they fall at, or below, the assay detection limit. Samples from males would lie at $\geq$ 80% B_0 in an analytical region of low estimation precision.

A preliminary quality control test showed DPC's current salivary T protocol inadequate. A parallelism check consisting of duplicates of a 6-step serial dilution [1:1.25–1:40, in 0.05 M phosphate buffered 0.15 M saline (PBS), pH 7] of a saliva pool collected in-house from young adult male volunteers was run along with duplicates of an identical serial dilution of the same pool spiked at each dilution with a constant 160 pg pure T/mL. Expected results for the simple dilution series are ~1–140 pg/mL (*cf.* 3,4), however, a nearly constant 54 ± 11 pg/mL (mean ± SD) was observed regardless of dilution factor; 16 other undiluted saliva samples from other young males measured 44 ± 23 pg/mL. The spiked saliva dilutions differed consistently from the simple saliva dilutions (190 ± 44 pg/mL observed; 160 pg/mL expected). As this is a direct assay with no sample

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purification involved, any competing steroids or matrix proteins, and their potential interferences, would be equally present in both dilution series. The recoveries (observed T/expected T) of the spiked material support DPC's data on antibody specificity. The low unspiked T values imply assay sensitivity for T in saliva using DPC's protocol was not as low as the 10 pg/mL inferred from the 2 pg/tube limit in the unmodified assay and the 200 μL sample volume of the modified assay. Indeed, we observed an initial limit of detection (defined as the concentration of T corresponding to B_0 − 2 SD of B_0) of 16.6 pg/mL. A nonlinear regression curve of cpm on log T (pg/tube) for standards in this preliminary assay was:

$$cpm = -1{,}869[log\ T]^2 - 592[log\ T] + 17{,}521$$

($r^2 = 0.99$). Biorad Lyphochek control sera (series 60000) diluted 1:20 in water, fell in the anticipated diagnostic ranges.

To improve the ability of the DPC kit to adequately assay even young males' salivary T, we increased sample volume to 400 μL of saliva, bulked the 20-fold–diluted serum standards with an additional 200 μL of PBS, and retained the remainder of the DPC salivary protocol, including tracer volume. Tracer constitutes 71.4% of final assay volume in our protocol versus 83.4% in DPC's protocol. A second parallelism check incorporated duplicates of a 6-tube serial dilution of T in charcoal-stripped saliva (final levels = 0, 12.5, 25, 50, 100, or 200 pg T/mL). The series was made by diluting a charcoal-stripped saliva pool derived from our in-house adult male pool to which 500 pg pure T/mL had been added with an identical pool of unamended charcoal-stripped saliva. Biorad Lyphochek standards diluted 1:20 in water were also included to help revalidate the assay for the larger sample volume.

The B_0 values from zero standards (no T) in diluted serum or in charcoal-stripped saliva agreed ($B_{0,1} \neq B_{0,2}$, $t = 1.367$, $p > 0.2$). The standard curve for the diluted kit standards was:

$$cpm = -1{,}972\ [log\ T]^2 - 331[log\ T] + 17{,}439$$

($r^2 = 0.99$), that for the serial dilution of T in stripped saliva was:

$$cpm = -1{,}955\ [log\ T]^2 - 852[log\ T] + 18{,}202$$

($r^2 = 0.99$). These can be superimposed on the curve of the earlier assay using DPC's saliva protocol and 1:20 diluted kit standards. This suggests that neither additional dilution of kit standards (1:140 final sample dilution in assay tubes in our protocol, 1:120 final dilution in the DPC protocol) nor changing the standard matrix from diluted serum to charcoal-stripped saliva was the primary factor leading to improved detection of salivary T with our modified assay.

Intra-assay CV for all sample duplicates was 11.3 ± 10.3% (across three assays); limit of detection was 16 ± 4 pg/mL (determined from either diluted kit standards or charcoal-stripped saliva dilutions). The increase in sample volume shifted responses for male samples from 80–100% of B_0 to 30–80% of B_0 and improved the CV on duplicated male saliva samples from 25.9% to 10.8% (based on the same 14 subject samples assayed with both DPC's and our protocols). Accuracy in the new assay was shown by an average spiking recovery of 106.9% for eight charcoal-stripped saliva samples spiked with 25–200 pg/mL of T and by an $r^2 = 0.98$ for estimated versus actual T for the entire series of 12 samples spiked with 0–200 pg/mL. Estimated levels of salivary T in 14 males measured using both protocols was 41 ± 22 pg/mL (range 17–103 pg/mL) with the DPC protocol, but 112 ± 46 pg/mL (range 47–198 pg/mL) with our modification; values from the two protocols were uncorrelated ($r^2 = 0.054$; testing $r = 0$, $t = 0.898$, $p > 0.2$). The newer estimates are more consistent with published levels (3,4). Values for 155 additional samples measured in duplicate under our protocol averaged 117 ± 64 pg/mL (range 20–345 pg/mL); only 2 of 171 male samples evaluated in this protocol registered below assay sensitivity, indistinguishable from any zero standards. Biorad standards replicated expected values.

The DPC kit and salivary protocol may work with a higher specific activity tracer, with antibody coated tubes of lower load capacity, or with a two-step protocol using immunoextraction of analyte from sample followed by competition with tracer. During our studies they proved inadequate to measure T in the recommended 200 μL of raw saliva. As matrix effects do not appear the most important factors in our improving performance of this assay, pushing analyte mass above the minimum needed to generate a proportionate signal with the existing kit materials is more likely the key variable.

We suggest clinical or psychological investigators intending to measure salivary T with this DPC assay increase salivary sample volume to ≥ 400 μL. Salivary T in female, immature, or elderly subjects (3–6) is inaccessible without modification of DPC's protocol as results would fall at, or very near, B_0. Here, the DPC assay will require revalidation using up to 1 mL of saliva as sample. Alternatively, a reconstituted solid-phase or organic extract of saliva, a higher specific activity tracer, or a two-step protocol should be used and the assay revalidated accordingly. DPC's validation holds well under our modification and would probably be robust to the changes just suggested for low level samples. In all cases, a normal set of quality control checks should be run and quality assurance values reported whenever the assay is used for saliva. Unfortunately, these data are often missing in earlier behavioral studies (*e.g.* 7), which prompts questions concerning adequate assay control in those studies as their central conclusions often depend on modest changes in levels of hormone.

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