Saliva and stool testing provide significant laboratory insight into stress, hormonal balance, and gastrointestinal health. To insure accuracy, it is vital, prior to testing, to screen for and avoid substances and practices that might skew, or otherwise influence, test results.

Sometimes, samples returned to Diagnos-Techs™ are not tested due to an error in collection on the part of the patient. In other cases, results obtained by saliva or stool testing are abnormally low or high due to an interfering food or medication. Our Client Services and Medical Support departments frequently are called by providers regarding cautions to be observed prior to testing. In order to avoid the time delay, frustration, and, sometimes, extra expense generated by improper test submission or inadvertent contamination, what follows is a general review of recommendations for patients and providers to optimize specimen collection for the highly sensitive testing offered by Diagnos-Techs™.

Initially a clinician should select a test specific for the diagnostic or screening purpose intended. Consider whether the test is to investigate a new patient previously untested, or whether it is a follow up on the status of a patient undergoing treatment. For a new patient, a clinician can opt for either of two investigatory approaches: (1) establishing the baseline immunologic, hormonal, or intestinal status of the patient in order to establish a comprehensive treatment plan; or (2) testing the patient just as he/she presents to discern all the factors, known and inadvertent, endogenous and exogenous that may affect the patient’s actual status. The former involves stopping all exogenous factors, both known and unknown, which could influence the patient’s status; this cessation also must be for a sufficient length of time. It is important to recognize that some influencing factors are a regular part of a patients routine and cannot, will not, or probably should not, be stopped (e.g., mesalamine [Asacol®] for Crohn’s disease, or steroid inhaler for asthma).

Once a plan of action is decided upon, the clinician and the patient should be aware of some common testing pitfalls. These pitfalls, whether for saliva or stool samples, fall roughly into one of three categories: test selection, specimen collection, and interfering factors.

Continued on page 2.
Test Selection

Certain tests are contraindicated under some circumstances.

- **Cycling Female Hormone Panel (FHP™)**—This panel is not advised for menopausal women (defined as no menstrual period for one year or more). The menopause may be natural, or surgical. The latter category includes total and partial (one or both ovaries retained) hysterectomies, and uterine lining ablation procedures (no endometrial lining to slough at the end of the cycle). Although a few women have a vague sense of their cycle after these procedures, an accurate knowledge of the individual cycle is best for the proper timing of the vial collection. In the cases of partial hysterectomy and uterine lining ablation, hormonal rhythm may be identified by tracking a woman’s basal body temperature for three months (cycles). Graphs for plotting basal body temperatures can be downloaded from our website, www.diagnostechs.com. This strategy can identify the tell-tale temperature rise observed frequently with ovulation.

  The time and effort involved with basal temperature graphing might be inconvenient for some patients. Under these circumstances, a less ideal alternative is the Perimenopausal Panel (PeriM™) which involves two collections about two weeks apart. If ovulation occurs, the timing can be identified and an appropriate collection schedule worked out.

- **Birth control**—These medications are designed to prevent ovulation, such that no pre-ovulatory estradiol or post-ovulatory progesterone surges occur. Therefore, if a woman is using birth control, the FHP™ panel is of little value. To check the effectiveness of a birth control regimen, the single-vial Postmenopausal Panel (PHP™) can be performed around day 21 to check for any post-ovulatory progesterone elevation (elevated progesterone signals ovulation, and, thus, an ineffective regimen). In view of the often prolonged after effects of birth control usage, the Cycling Female Hormone Panel (FHP™) is not recommended sooner than 3-4 cycles after birth control cessation.

- **SIgA based results**—Many of the tests reported by Diagnos-Techs rely on detection of SIgA antibodies to the antigens being tested. These tests include both food intolerance and parasitic infection. The body cannot mount an adequate SIgA antibody response if it has insufficient IgA. This insufficiency may be congenital (about 3-4% of the population is affected), or from inadequate production due to chronic stress or inflammation. For results to be meaningful, any SIgA-dependent tests (e.g., food intolerance or parasite testing) should be accompanied by a separate salivary total SIgA analysis.

- **Follow-up treatment based on SIgA antibodies**—Unlike several other immunoglobulins, SIgA antibody production recedes after the inciting antigen is removed, (e.g., food restriction, or antimicrobial/antiparasitic treatment). The antibodies already present slowly dwindle over time. Therefore, follow-up specific SIgA antibody testing following antiparasitic therapy or dietary restrictions (e.g., a gluten-free diet) to assess treatment efficacy should be delayed 3-4 months after treatment for the results to be valid.

Specimen Collection

Tests returned to the laboratory can be unacceptable for several reasons.

- **Incomplete documentation**—To ensure accurate reporting, the requisition forms must be completed, and the patient’s name and date of birth must appear on each vial. Moreover, the correct vial for the test must be used. Details are spelled out in the instructions. If the appropriate vial is unavailable for any reason, a proper substitute usually is available from a Flexi Matrix™ test kit. Guidance is available from Client Services.

- **Insufficient saliva**—When an absorbent roll is involved (e.g., with ASI/TAP™ test kits) it must be saturated completely; the roll should be held under the tongue until the mouth refills with saliva before returning the roll to the appropriate vial. Absent an absorbent roll, saliva should be drooled passively into the proper vial until it is one half to three quarters filled with liquid saliva (no froth). Salivary flow can be encouraged by making a chewing motion (with or without paraffin wax), or by smelling a lemon, pickle juice or vinegar.

- **Appropriate Protocol Choice for Testing Hormones**—
  
  - To establish baseline values for a patient, there should be no exogenous hormone ingestion, orally or sublingually, for at least 3-4 weeks prior. This caution applies to all topical hormones such as cortisone, estrogen, or testosterone, with the exception of topical progesterone. Progesterone creams are absorbed into subcutaneous lipid cells and
stored there. After cessation of progesterone cream use, it often takes several months for the lipid stores of progesterone to deplete.

• To monitor hormone therapy, testing can be carried out immediately—with certain precautions. Transdermal medications and oral medications should be avoided the day before, and the day of collection (until collection is complete). Sublingual hormone medications (e.g. progesterone or DHEA) potentially can contaminate the saliva and produce spurious results. Therefore, all sublingual hormones should be continued at the same dosage, but swallowed with water (taken orally) starting three days prior to testing. Like all oral hormones, they must be avoided the day prior to and the day of specimen collection.

Interfering Factors

Both hormone and GI testing may be perturbed unwittingly by ingesting or using certain medications or oral products, or through improper timing of the collection.

• Hormone testing—(Please see Table 1)

Exogenous Cortisone and related compounds can throw off cortisol values in the ASI/TAP™ panels. Sources of cortisone include cortisone ointments (e.g., anti-itch cream or hemorrhoid creams), steroid inhalers, and cortisone eye drops. All non prescribed progesterone intake should be stopped since progesterone not only is an antecedent of cortisol but can cross-react with cortisol during testing.

DHEA is commonly present in over the counter (OTC) supplements, alone or in formulations. It is often prescribed or self-administered orally or sublingually. DHEA may be added to commercial products such as anti-wrinkle creams and soaps.

Progesterone may be prescribed, but note that it is added commonly to many anti-aging skin products and cosmetics; and it is available as OTC creams. All progesterone intake, especially in cosmetic and anti-wrinkle creams, must be inquired about and taken into account when interpreting results.

Exogenous Testosterone generally is applied as an ointment or gel; thus passive transfer to a partner may occur readily. Applications should be avoided for 3-5 days prior to testing either partner to insure unbiased results.

Estrogens are present in many OTC supplements and should be inquired after. They are also added to some cosmetics.

• GI testing—can be interfered with by a number of factors. Submission of a stool sample with a low quantity of solids (e.g., diarrhea stool) can result in an insufficient amount for testing. Testing should be delayed at least 10-14 days following lower bowel studies (e.g., barium enema or sigmoidoscopy/colonoscopy) to provide ample time for the bowel flora to repopulate and rebalance after the pretest bowel preparation). Some other important considerations are presented in Table 2.

<table>
<thead>
<tr>
<th>Hormone Exposure Cautions (Exogenous causes)</th>
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<tbody>
<tr>
<td><strong>Cortisone</strong>—skin/ hemorrhoid creams; eye drops; inhalers; non-prescribed progesterone</td>
</tr>
<tr>
<td><strong>DHEA</strong>—Anti-wrinkle creams; soaps; OTC self administration</td>
</tr>
<tr>
<td><strong>Progesterone</strong>—Anti aging and anti wrinkle creams; cosmetics; OTC self supplementation</td>
</tr>
<tr>
<td><strong>Testosterone</strong>—Hormone supplement gel/ointments (possible transfer to partners)</td>
</tr>
<tr>
<td><strong>Estrogen</strong>—Creams; OTC supplements; cosmetics</td>
</tr>
</tbody>
</table>

Continued on page 4.
Optimizing Test Accuracy
continued from page 3.

Collection Timing
Appropriate timing of certain collections is essential for optimal results.

- **Hormones**: Much like cortisol, the production of most sex hormones follows a diurnal rhythm. This must be taken into account. The maximum hormonal output for most male and female hormones is present after awakening from sleep. When assessing a patient’s peak hormone values, collection optimally should take place within 2-3 hours of waking. This is particularly important when assessing testosterone for possible hypogonadism (andropause) and female hormone levels at menopause. For serial collections such as those requested for the Female Hormone Panel (FHP™), consistent early morning collections might be a challenge. Under these circumstances, collections can be done later in the day as long as they are done within 1-2 hours at the same time of the day. Valid comparisons and an accurate plot of the hormone changes during a complete cycle can be carried out.

- **GI Collection**: Most of the testing assayed in the samples collected for the regular and expanded GI Panels (GI-1™ and GI-02™) can be processed as long as they are received within 7 days of collection, with the exception of the stool cultures for bacteria and fungi (vial A). To avoid overgrowth with the potential chance for misdiagnosis, vial A must reach the lab within 3-5 days of collection. To accomplish this goal, the A vial should be collected last, just prior to shipping (Sunday collection is optimal). Kits should be shipped Monday or Tuesday. Should this be problematic due to weekend interruption or difficulty with UPS pickup, the kit can be refrigerated temporarily (never frozen).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Pitfalls in GI Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product Category</strong></td>
<td><strong>Avoid For 3-4 Days Prior And Day Of Collection</strong></td>
</tr>
<tr>
<td>Anti-gas</td>
<td>Activated charcoal</td>
</tr>
<tr>
<td><strong>Examples</strong>:</td>
<td></td>
</tr>
<tr>
<td>Anti-diarrheal, GI upset</td>
<td>Bismuth products, kaolin, opiates</td>
</tr>
<tr>
<td><strong>Examples</strong>:</td>
<td>Kaopectate, Pepto Bismol</td>
</tr>
<tr>
<td>Laxatives/stool softeners</td>
<td>Enemas, psyllium/flaxseed products, gums, mineral oil, Milk of magnesia, Bulk laxatives</td>
</tr>
<tr>
<td><strong>Examples</strong>:</td>
<td>Fleet, Metamucil</td>
</tr>
<tr>
<td>Natural digestive aids</td>
<td>Digestive enzymes, Bromelain, papain, garlic, essential oils</td>
</tr>
<tr>
<td><strong>Examples</strong>:</td>
<td></td>
</tr>
<tr>
<td>Anti-microbials/GI cleansers</td>
<td>Prescription antibiotics; prescription and natural Antibacterials, antifungals And anti-parasitics</td>
</tr>
<tr>
<td><strong>Examples</strong>:</td>
<td>(Physician Discretion)</td>
</tr>
<tr>
<td>Anti-inflammatory/Pain medications</td>
<td>Steroids (prednisone, etc..)</td>
</tr>
<tr>
<td><strong>Examples</strong>:</td>
<td>(Physician Discretion)</td>
</tr>
</tbody>
</table>

Summary
Careful attention to test selection, specimen collection, and test interfering factors can assure accuracy and meaningful results. Delay, frustration, and associated expense can be avoided. High quality and timely results will be assured.
Insulin Testing at Diagnos-Techs™

John J. White, MD CM and Brandy Webb, ND

Diagnos-Techs™ offers salivary insulin testing as part of the Adrenal Stress Index™ (ASI™) panel and the Carbohydrate Challenge Test™ (CHO™). The initial portion of each panel is an assay for fasting insulin. After an overnight fast, insulin values expectedly should be negligible. Our reference range for fasting insulin is 3-12 uIU/mL. Inasmuch as this assay is less precise at low levels, we report any values lower than the listed range as <3 uIU/mL. Normal values should be expected to fall in or below that range; this is the reason that patient values of <3uIU/mL are not designed as “depressed”.

The second insulin value in the ASI™ panel is sampled from the second, or “noon”, vial. To assess a patient’s dietary habits, no dietary attention is explicitly required. However, many practitioners prefer to use the second sample to assess the endocrine capability of the pancreas. For this purpose, the patient must ingest a carbohydrate-rich meal (consisting of 75g of carbohydrates) sixty minutes prior to taking the noon sample. Detailed food item recommendations and additional information are included in the ASI™ Specimen Collection Instructions pamphlet. The resulting value represents the capabilities of the Islets of Langerhans in the pancreas to respond to a GI and serum carbohydrate load.

The Carbohydrate Challenge Test™ (CHO™) from Diagnos-Techs™ is available to evaluate more thoroughly insulin sensitivity and glycemic dysregulation, and as a follow-up to aberrant values discovered on the ASI™ panel. The CHO™ is comprised of blood glucose readings plus saliva cortisol and insulin measurements. The patient is instructed to collect a fasting saliva specimen, which is assessed for cortisol and insulin; followed by additional specimens at 1-, 2-, and 3-hours after consuming a carbohydrate-rich meal. To provide a more comprehensive picture, the patient is instructed to provide blood glucometer readings (similar to diabetics) for the fasting ½-, 1-, 2-, and 3-hour postprandial states. The glucose measurements, along with accompanying heart rate readings, are recorded on the provided form, which is returned with the saliva samples. A comprehensive assessment of the patient’s glucose-stress regulatory and counter-regulatory status is returned to the provider.

Salivary insulin assays provide a fuller understanding of carbohydrate metabolism vis-à-vis the adrenal stress response. The ASI™ and CHO™ provide relatively atraumatic and economic means to examine and quantitate possible aberrancies in glucose metabolism.

Jennifer M. Kooken, PhD

Dr. Kooken received her BS degree in Biology from Syracuse University where she also participated in Division I track and field. She attended the University of Rochester School of Medicine’s Post Baccalaureate Research Education Program (PREP); there in the lab of Dr. John Rose she worked on the expression and characterization of recombinant Human Herpes Virus 6b (HHV-6b) proteins for use in diagnostic assays. Following this, Dr. Kooken worked at a pharmaceutical company in Cambridge, MA developing ELISA based assays to evaluate drug molecules. She earned her PhD at the University of South Carolina, as an Alfred P. Sloan scholar, while completing research at Pacific Northwest National Laboratories with a Science and Engineering fellowship. Her research focused on the use of top-down and bottom up proteomics on Bacillus and Staphylococcus species. This culminated with her dissertation focused on the development of proteomic characterization and speciation techniques utilizing tryptic peptides with MALDI-TOF MS and LC-ESI MS-MS.

At Diagnos-Techs™, Dr. Kooken is participating in the active laboratory research efforts utilizing both GS and LC MS-MS.
Ever increasingly, newer hormonal therapies are being advocated for hypotestosterone states such as andropause, the male equivalent to menopause in a woman. To anchor intelligent therapy and avoid a number of pitfalls to meaningful testing, three areas merit consideration.

I. Shortcomings of Current Serum Testosterone Testing:

Serum testosterone assays alone are commonly relied upon. Basically, it must be remembered that all hormones in the blood are in two forms—bound to hormone binding globulins (90-97%), and, unbound (free or available). The unbound forms traverse the capillaries into the tissue fluid to affect their target cells. Although any measured free hormones in the serum are presumed to enter the tissue fluid, their constant interchange with binding globulins in the serum calls any attempt at precise assay into question.

These shortcomings have been recognized by The Endocrine Society for testosterone, the hormone with the lowest free fraction (<3%). In 2008, The Endocrine Society issued a position statement stating that “the manner in which most [serum] assays for TT [total testosterone] and FT [free testosterone] are currently performed is decidedly unsatisfactory.”¹ Recognizing that “important discrepancies and inconsistencies in measurements are widespread,” in 2010 The Endocrine Society paired with the Center for Disease Control (CDC), other clinical societies, and commercial laboratories to endorse “accuracy-based [serum] testing of testosterone and calibration of all methods traceable to a single high-level reference material.”² These methods have been developed by the CDC using mass spectrometry and offered to all interested parties. This process is ongoing. A clinician opting for serum testosterone testing should ensure that the laboratory conforms to the CDC standards.

This standardization program, itself, has a major drawback. The “simple, high level reference material” used for standardization consists of total serum testosterone, i.e., both bound and unbound hormones together. The actual level of free or active testosterone can only be assumed.

Saliva has minimum binding proteins and is, essentially, tissue fluid. Salivary testosterone measurements thus represent the free or active form of testosterone. They have been shown to have significant correlation with free serum testosterone measurements (Fig 1)³, using radio immunoassay (RIA) techniques. RIA techniques are not used frequently since radiation hazard is inherent and they are costly.

Diagnos-Techs™ utilizes the equally as accurate enzyme linked immuno specific assay (ELISA) methodology. Final verification of our results currently are in progress utilizing our new mass spectroscopy capabilities, in conjunction with Georgetown and Johns Hopkins medical centers. Serum free testosterone assay is expensive, and difficult to carry out; consequently, it usually is done only for purposes of laboratory investigation. The only available assays for free testosterone are salivary testosterone measurements. Aside from being more accurate and clinically meaningful, they are non-invasive, private, and inexpensive.

II. A Complete and Thorough Evaluation:

Currently, clinical investigation starts with a simple serum testosterone assessment. Often, therapy is recommended based on this value alone, despite its inherent shortcomings, and the incompleteness of the evaluation. This may occur due to lack of appreciation of factors, other than hypogonadism, which can lead to low levels of testosterone, and to a sense of urgency to provide treatment. Further serum

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Figure 1

Significant correlation ($P = 0.0001$) between salivary testosterone (Sal-T) and serum free-testosterone (Free-T) levels was observed in 72 male subjects.
investigations involve additional time, and are costly.

The Androgen Pathway Panel (MHP™) from Diagnos-Techs assesses comprehensively the pathway of testosterone metabolism and pituitary controlling factors (LH and FSH), all at the same time, and from a single sample. (Fig 2) DHEA, progesterone, and androstenedione, the major antecedents of testosterone, are measured. Deficiencies or excesses of these hormones influence testosterone values. In addition to testosterone, the less common but more potent derivative, dihydrotestosterone (DHT), is also measured. The two pituitary controlling gonadotrophins assayed, FSH and LH, are an assessment of hypothalamic/pituitary influence upon testosterone/DHT production (this assessment is recommended by most endocrinologists). Finally, in view of the increasing propensity in older men for aromatization of testosterone to the feminizing hormone, estradiol (E2), both estril (E1) and estradiol are measured. This very complete and thorough laboratory investigation of suspected andropause is carried out non-invasively and economically, in the privacy of one’s own home.

The retail charges (the cost of each test ordered individually) for the nine hormones tested in the Androgen Pathway Panel (MHP™) total approximately $1150.00 in a representative serum testing laboratory; at Diagnos-Techs™ these nine hormone tests are $390.00. When ordered as a panel, the cost to the patient is $120.00, approximately $13.33 per hormone tested.

**III. Proper Timing of Androgen Testing:**

Like most hormones (best known is the cortisol circadian rhythm), testosterone has circadian rhythm. Values are highest in the morning, falling off during the remainder of the day. Therefore, in order to determine a man’s highest testosterone values, sampling ideally should be done in the morning—after awakening and no later than 10 AM. Later sampling only measures the ebbing testosterone, and is not representative of maximum values. Should androgen treatment be elected based on low values from samples collected later in the day, over-treatment may result. This can lead to aromatization of the resultant elevated testosterone to estradiol, with consequent feminization.

In summary, a complete salivary androgen assay provides accurate assessment of the free or active testosterone, plus an in depth measurement of the several factors determining testosterone metabolism and ultimate values. For accuracy, sampling must be carried out in the morning. Using the Androgen Pathway Panel (MHP™) from Diagnos-Techs™, a complete, economic, and meaningful evaluation of suspected hypogonadism in men can be obtained.

**References**

Storage & Mailing Instructions for All Specimens

- Ship samples on the same day as last sample collection (preferred).
- If not possible, refrigerate samples and ship within 3 days. No ice bags are required during shipping.
- Write the patient’s name and address on the outside of the box.
- Include all samples, test form and, if applicable, a check or a copy of the front and back of insurance card. Please be sure to seal the box with clear tape OR the UPS shipping label (U.S. only).

US Domestic:
- Deliver completed test kit box to any UPS location. www.UPS.com/dropoff. Return shipping to Diagnos-Techs™ is PRE-PAID. Kits will arrive within three business days of shipment.

International:
- Delivery charges apply. Visit our website for access to discounted return shipping via UPS. Deliveries can also be made Monday through Friday via a private courier of your choice. International deliveries should be addressed to the physical address only, as noted to the right. Do not address to the PO Box.