ACQUITY LABS
...empowers to live well

APPLICATION NOTES

Development, Validation and Application of Chemiluminescent Immunoassay Method for Hormone Analysis Compatible With VAMS® Remote Specimen Collection Technology

Yashaswini Sammeta, Dr. Aksah Navilebasappa
515, 3rd Floor, 4th Main, 1st Cross, 1st Stage, 4th Block, HBR Layout, Bengaluru 560043, Karnataka, India

ABSTRACT

This technical brief presents methods for measuring 12 different Hormone concentrations (Anti Mullerian Hormone (AMH), Follicle Stimulating Hormone (FSH), Thyroid Stimulating Hormone (TSH), Total testosterone (T), Prolactin, Free Thyroxine (FT4), Total Thyroxine (TT4), Estradiol (E2), Progesterone, Luteinizing Hormone (LH), Dehydroepiandrosterone (DHEAS) and Cortisol) in patients from blood samples collected remotely using Mitra® microsampling devices, which contain VAMS® microsampling tips. Measurement of hormones from samples taken by individuals at home and sent to a laboratory for analysis is an ideal solution for remote monitoring and access to larger participant pools for population health surveys. This is especially true during a viral pandemic, when vulnerable people cannot safely travel to a clinic for a blood draw. Using examples from studies that have compared traditional venipuncture sampling versus VAMS, we show that with more research focused on stabilizing blood microsamples, remote blood collection with VAMS for accurate hormone measurement is feasible.

INTRODUCTION

VAMS is a novel approach to obtaining a precise volume dried blood sample (typically from a finger-stick) for quantitative bioanalysis that overcomes the area bias and homogeneity issues associated with conventional dried blood spot (DBS) sample when a sub punch is taken. By simply touching the VAMS sampling tip to a drop of blood (see figure 1) it absorbs a fixed volume of sample (~10, 20 and 30µL,) in 2–4 seconds with less than 5% volume variation across the hematocrit range of 20–70% with low tip-to-tip variability. (1, 2)

Figure 1. Mitra device containing VAMS® sampling tips absorb a precise amount of blood from a finger-stick.

After the sample is collected, in the clinic or field, it can be packaged in a specimen shipping bag containing desiccant and shipped to the bioanalytical laboratory for analysis. For the purposes of this application note a panel of hormones were analyzed:

Anti-Müllerian hormone (AMH) is an important biomarker of ovarian reserve that has the potential to facilitate new research on fecund ability, infertility and reproductive aging. AMH is a glycoprotein dimer produced by granulosa cells from small pre-antral and antral follicles in the ovary, and it is hypothesized to inhibit the recruitment of primordial follicles into the pool of growing follicles. Studies suggest that AMH represents a useful measure of ovarian reserve since it plays an important role in early-stage follicle development. (3)

Thyroid-stimulating hormone (also known as thyrotropin, thyrotropic hormone, or abbreviated TSH) is regarded as the primary biomarker for evaluating thyroid function. TSH is secreted throughout life but particularly reaches high levels during the periods of rapid growth and development, as well as in response to stress. (4)

Testosterone (T) is the primary sex hormone and anabolic steroid in males. Measurement of testosterone is important in the diagnosis and monitoring of males with hypogonadism. In females, T and androstenedione (A4) measurement is useful for identifying patients with androgen excess such as polycystic ovary syndrome (PCOS). (5, 8, 10, 11)

Prolactin levels may be checked as part of a sex hormone workup, as elevated prolactin secretion can suppress the secretion of follicle stimulating hormone and gonadotropin-releasing hormone, leading to hypogonadism sometimes causing erectile dysfunction. Measurement of prolactin concentrations in serum of lactating amenorrheic women provides a sensitive predictive index of returning menstruation and fertility. (6)

Thyroxine (T4) hormone is made by thyroid gland in response to thyroid stimulating hormone. T4 is found in the body in two forms: free T4 and bound T4. Free T4 travels into body tissues that use T4. Bound T4 attaches to proteins that prevent it from entering these tissues. More than 99% of T4 is bound. T4 helps to control how our body store and uses energy. The thyroid hormones also help control other processes like breathing, heart function, cholesterol level, menstruation etc. Thyroxine measurement also can show If our thyroid gland is overactive or underactive. (7)

Estradiol has several functions in the female body. Its main function is to mature and then maintain the reproductive system. During the menstrual cycle, increased estradiol levels cause the maturation and release of the egg, as well as the thickening of the uterus lining to allow a fertilized egg to implant. The hormone is made primarily in the ovaries, so levels decline as women age and decrease significantly during menopause. In men, proper estradiol levels help with bone maintenance, nitric oxide production, and brain function. While men need lower levels than women, they still require this important hormone to function well. (8, 10)

Progesterone (P4) is an endogenous steroid involved in the menstrual cycle, pregnancy and embryogenesis of humans. It is a crucial metabolic intermediate in the production of other endogenous steroids, including sex hormones and the corticosteroids, and plays an important role in brain function as a neurosteroid. (8, 10)

Luteinizing hormone (LH) is an important hormone both men and women produce. This hormone is known as a gonadotropin, and it affects the sex organs in both men and women. For women, it affects ovaries, and in men, it affects the testes. LH plays a role in puberty, menstruation, and fertility. LH is a hormone that's produced in the pituitary gland. The pituitary gland is located at the base of the brain, and it's roughly the size of a pea. If you're a woman, LH is an important part of your menstrual cycle. It works with **Follicle-stimulating hormone (FSH)**, which is another gonadotropin made in the pituitary gland. FSH stimulates the ovarian follicle, causing an egg to grow. It also triggers the production of estrogen in the follicle. The rise in estrogen tells your pituitary gland to stop producing FSH and to start making more LH. The shift to LH causes the egg to be released from the ovary, a process called ovulation. In the empty follicle, cells proliferate, turning it into a corpus luteum. This structure releases progesterone, a hormone necessary to maintain pregnancy. If pregnancy doesn't occur, the levels of progesterone drop off and the cycle begins again. If you're a man, your pituitary gland also produces LH. The hormone binds to receptors in certain cells in your testes called Leydig cells. This leads to the release of testosterone, a hormone that's necessary for producing sperm cells. The amount of LH in your blood can indicate underlying problems associated with a variety of reproductive health issues. (9)

Dehydroepiandrosterone (DHEA), a hormone produced by the adrenal glands, is the precursor for the production of estrogens and testosterone, and is therefore normally present in significantly greater quantities than all the other steroid hormones. It is mostly found in the circulation in the form of its sulfate ester, DHEA sulfate (DHEA-S), which is usually measured in the sample because its circulating levels are higher and more stable. Its production is highest in the late teens to early 20s, and declines gradually with age in both men and women. Levels of DHEA-S reflect adrenal gland function. (10)

Cortisol is a steroid hormone. It is produced by the zona fasciculate of the adrenal cortex in the adrenal gland. It is released with a diurnal cycle and its release is increased in response to stress and low blood glucose concentration. It functions to increase blood sugar through gluconeogenesis, to suppress the immune system, and to aid in the metabolism of fat, protein and carbohydrates. It also decreases bone formation. (11)

An obstacle to the measurement of hormones in community-based studies, or studies requiring multiple blood draws, is the requirement for venous blood. Venipuncture blood draws are costly, invasive and must be performed by a trained phlebotomist in close proximity to a facility where blood samples can be centrifuged, separated and frozen. Dried samples represent a minimally invasive alternative. The participant's finger is cleaned, pricked with a sterile, disposable lancet and collected by Mitra® device. A ma-

jor advantage of Mitra sampling is that it is relatively painless and noninvasive, and can be self-collected in the participant's home or other non-clinical setting. The simplified logistics of Mitra® sampling allow investigators to collect blood from large numbers of participants in diverse research settings. (12)

OBJECTIVE

The objective of this study was to evaluate whether adjustments to the sample protocol would allow combining the simplicity of Mitra-based home sampling with clinically relevant results.

MATERIALS AND METHODS

a. Overview

To study whether extracts from dried VAMS tips could be interfaced with the Chemiluminescent immunoassay. A comparison was made between dried VAMS samples (20 µL *4) and 2 mL of fresh blood collected in serum separator tubes under various conditions outlined below. First, an extraction optimization was carried out. Following this, VAMS samples were tested for temporal stability and various stabilization conditions were examined. A sample 15 % assay bias criteria, compared to the wet sample, was applied as a pass/fail for each test.

b. Sampling

2 mL of whole blood was drawn into a serum separator tube and was left to stabilize for thirty minutes at room temperature followed by centrifugation to separate serum for analysis. Mitra devices containing VAMS tips were sampled into the tubes where care had been made to ensure the blood was homogeneous by gently inverting the closed tube before each collection. Mitra device sampling followed Neoteryx collection kit instructions, where tips were allowed to touch the blood, but not fully immerse it, and was to be sampled by pointing the sampling tips downwards toward the floor. The blood was allowed to saturate the device tip. The tip was held in this position for another two seconds, and then gently removed from the blood to dry for 2 hours. Dried samples were divided into two sets and were stored at 4˚C and 30˚C each in a temperature-controlled cabinet until analysis. One third of sample from each set was processed immediately, one third was stored for 4 days and one third was stored for 7 days.

c. Optimization and Verification of Extraction Methodology

Extraction of the dried blood on VAMS tips was performed by removing those tips from the Mitra device and placing it in a 2 mL Eppendorf Tube® containing 200 µL of extraction solution. The tips were then exposed to several extraction conditions: Vortexing and sonication for different time intervals and temperatures. The result obtained showed better extraction when both vortexing and sonication at room temperature was employed. Once the extracts were prepared, they were treated the same as wet samples on the analyzer and analyzed within 60 minutes of extraction. The wet samples (no pre-treatment) and extracted VAMS samples were then analyzed per the manufacturer's instructions for various hormone analyses. The optimized conditions were then tested by comparing serum to dried blood VAMS samples from 50 volunteers.

RESULTS AND DISCUSSION

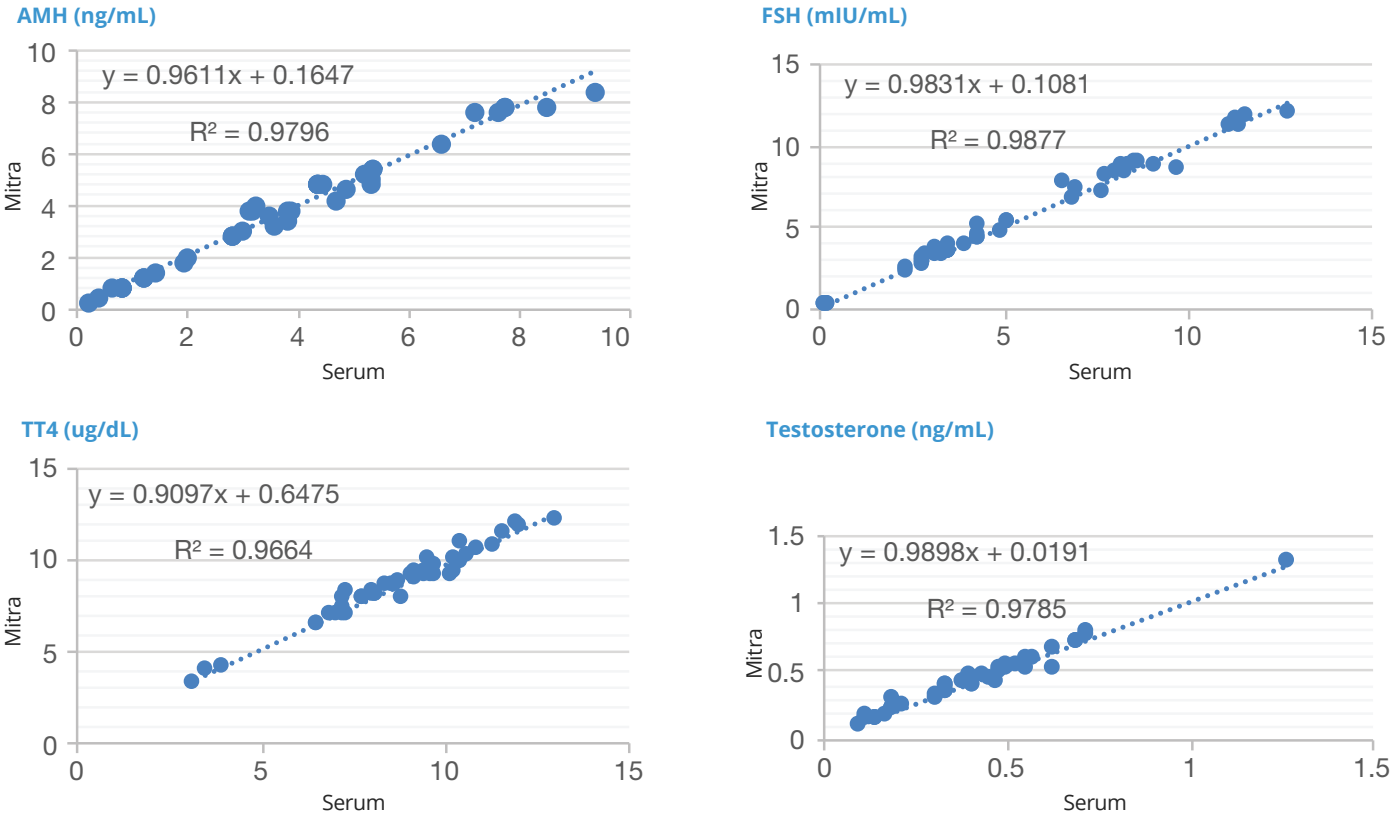
For each specimen, values obtained at the day of collection were used as the reference. Analysis was performed using an analytical method, for which reference values and decision limits for appropriate clinical situation are defined. Results for all hormones as shown in the below table were within the recommended concentration interval.

As seen in the plots below, there was a strong agreement between concentrations measured in VAMS samples and serum samples across the entire assay range for all the hormones. No statistically significant difference was found for all the hormones between specimens measured on the day of collection and those stored at 4˚c and 30˚c for 7 days. Results for Serum and VAMS samples stored at 4˚C and 30˚C were not statistically different at Day 0, Day 4 and Day 7.

Hormone	Number of subjects	Hormone Concentration Range	Observed Deviation
AMH	50	0.73 – 16.05 ng/mL	Slightly lower value was observed for about 14 samples at 30˚C on day 7. But it remained within the clinically acceptable range.
FSH	**50**	1.27 – 19.26 mIU/mL	All samples had FSH levels within the reference range and no significant changes were seen between any storage time / temperatures
TT4	50	6.09 – 12.23 ug/dL	No significant change in concentration was noted at any of the storage times and temperatures.
Total Testosterone	50	Females: 0.1– 0.75 ng/mL Males: 1.75 – 7.81 ng/mL	Values of samples of two females rose between 4th day and 7th day at 30˚C. But none fell outside the upper limit of normal.
Prolactin	50	2.64 – 13.13 ng/mL	One sample out of 50 showed a decrease from 6.24 (at day 4) to 4.5 (at day 7) stored at 30˚C. While all other showed a minimal decrease of 10%.
FT4	50	0.54 – 1.24 ng/mL	No significant change in concentration was noted at any of the storage times and temperatures.
TSH	50	0.45 – 5.33 uIU/mL	Minimal change of about 7% on an average was seen between Day 4 and Day 7 in samples stored at 30 C.
E2	50	10 – 400 pg/mL	<10% of decrease was observed from Day 4 to Day 7 in samples stored at 30˚C.
Progesterone	50	0.14 – 2.06 ng/mL	Progesterone concentrations showed no substantial change.
LH	50	1.24 – 8.62 mIU/mL	LH hormone increased by 2.9% and 4.7% on Day 4 and Day 7 respectively in samples stored at 30˚C.
DHEAS	50	Females: 2 – 389 ug/dL Males: 5 – 630 ug/dL	DHEAS was highly stable at both temperatures and no changes were observed.
Cortisol	50	6.7 – 22.6 ug/dL	Cortisol was stable during the study period, regardless of storage temperature.

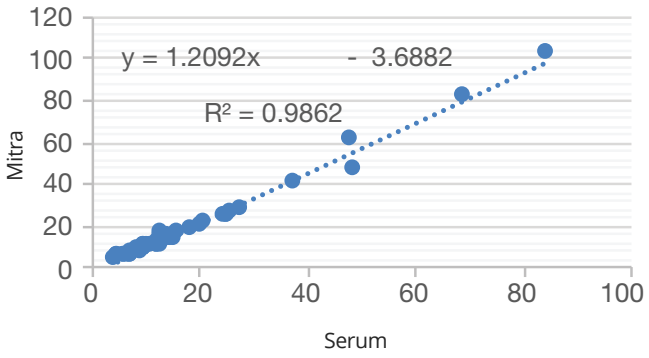
There was a strong agreement between concentrations measured in VAMS samples and serum samples across the entire assay range for all the hormones. Although the data for each was corrected to take into consideration the differences in dilution and the blood to serum partitioning ratio. No statistically significant difference was found for all the hormones between specimens measured on the day of collection and those stored at 4˚C and 30˚C for 7 days. Results for Serum and VAMS samples stored at 4˚C and 30˚C were not statistically different at Day 0, Day 4 and Day 7.

Figure 2. Correlations between Mitra and Serum for each of the hormones tested. All samples corrected with good precision. Biases seen between blood and serum were compensated using correction factors per analyte.'

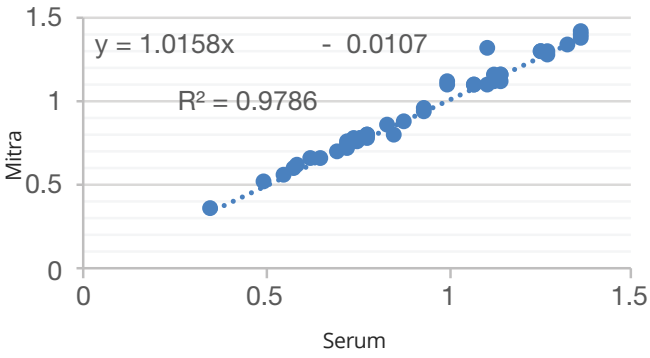


APPLICATION NOTES

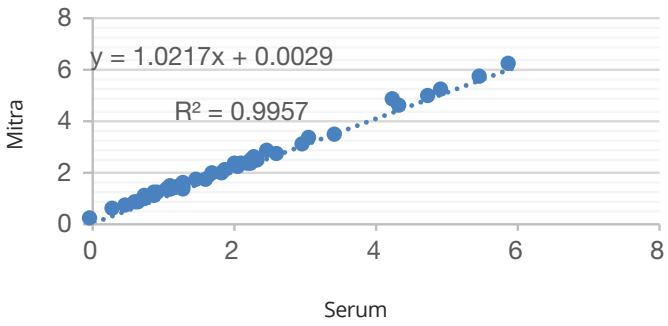
Prolactin (ng/mL)



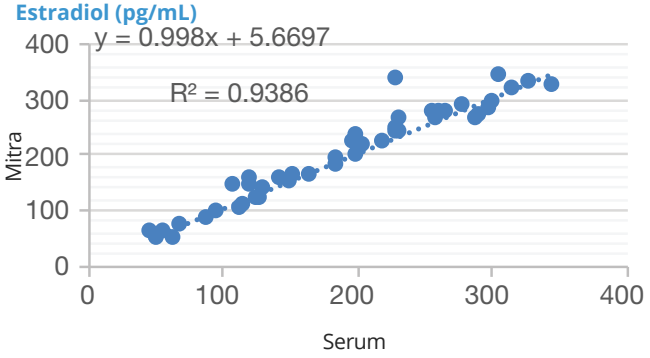
FT4 (ng/mL)



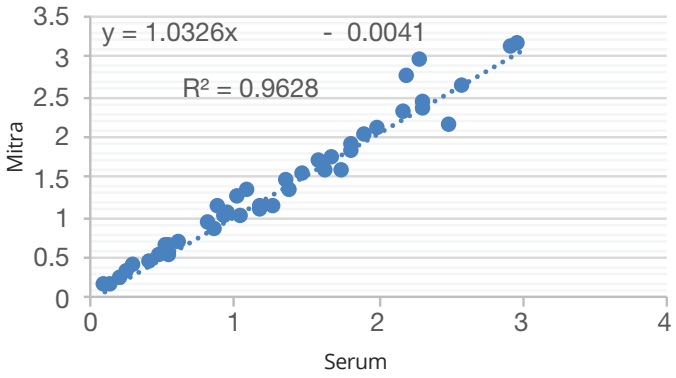
TSH (µIU/mL)



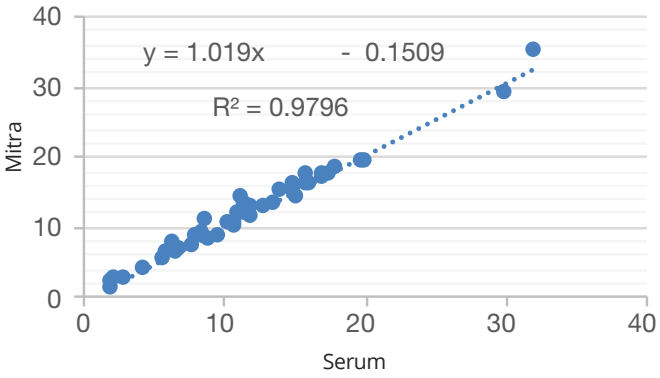
Estradiol (pg/mL)



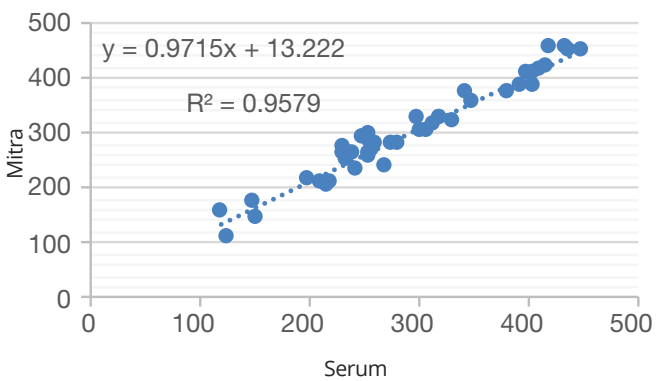
Progesterone (ng/mL)



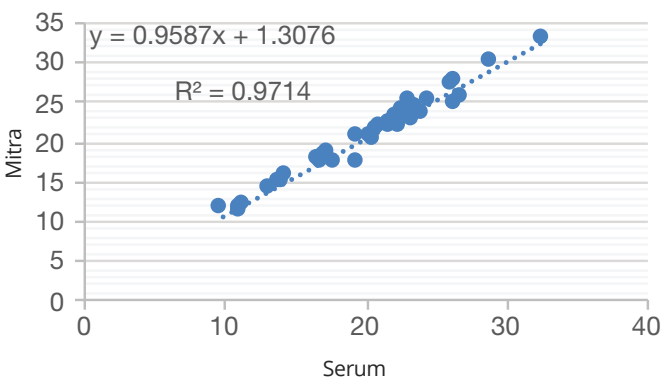
LH (mIU/mL)



DHEAS (µg/dL)



Cortisol (µg/dL)



Elevated temperature of 30 ˚C was employed in order to mimic the 'journey of a sample' from the point when home sampling is posted through the mail to the point when it arrives at a laboratory for final analysis. No consistent pattern of level variation with storage time and temperature was found. The concentrations of 12 hormones measured appeared stable up to 7 days at 30 ˚C when stored properly in a specimen shipping bag with desiccant. Also, the results show that when dried in the presence of desiccant, temperature did not seem to have a deleterious effect on the results. If possible samples have to be collected and sent to laboratory within 7 days. Mitra has proven to be a best platform for a remote patient-centric sampling option.

CONCLUSION

The results from this study show that high correlations can be observed between serum and dried blood from Mitra especially when correction factors were employed to compensate for the blood to serum partitioning ratio for each hormone. Moreover, the stability of the hormones on the Mitra samples were tested and shown to be sufficient to survive journeys between sampler collection and chemiluminescent immunoassay analysis in the laboratory. This study thus shows the utility of Mitra as a vehicle for collection of dried blood sample for the analysis of both the steroidal and peptide hormones tested.

REFERENCES

1. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. https://www.future-science.com/doi/full/10.4155/bio.14.310
2. Volumetric Absorptive Microsampling: A dried sample collection technique for quantitative bioanalysis. https://pubmed.ncbi.nlm.nih.gov/25058158/
3. Self-collected dried blood spots as a tool for measuring ovarian reserve in young female cancer survivors. https://doi.org/10.1093/humrep/dew114
4. Assays for thyroid stimulating hormone using dried blood spotted filter paper specimens to screen for hypothyroidism in older children and adults. https://journals.sagepub.com/doi/pdf/10.1258/096914103321610734
5. Quantification of testosterone, androstenedione and 17-hydroxyprogesteronein whole blood collected using Mitra microsampling devices. https://journals.sagepub.com/doi/abs/10.1177/0004563220937735
6. Radiommunoassay of prolactin in blood spotted on filter paper. https://academic.oup.com/clinchem/article-abstract/32/5/854/5652406
7. Total and free thyroxine and triiodothyronine: Measurement discrepancies, particularly in inpatients. https://doi.org/10.1016/j.clinbiochem.2014.06.007
8. Measurement of Gonadal Hormones in dried blood spot versus serum: Verification of Menstrual cycle phase. https://doi.org/10.1006/hbeh.2001.1657
9. Measurement of gonadotropins in dried blood spots. https://doi.org/10.1093/clinchem/40.3.448
10. Reproductive hormone measurement from minimally invasive sample types: Methodological considerations and anthropological importance https://onlinelibrary.wiley.com/doi/epdf/10.1002/ajhb.23535
11. Measurement of Cortisol and Testosterone in Athletes: Accuracy of Liquid Chromatography Mass spectrometry assays for Cortisol and Testosterone measurement in whole blood microspecimens. https://pubmed.ncbi.nlm.nih.gov/29985225/
12. What a drop can do: Dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. https://www.ipr.northwestern.edu/documents/working-papers/2006/IPR-WP-06-16.pdf