Comparing cortisol levels of saliva samples collected with the passive drool collection method with the Salivette® Cortisol

Anne Dubberke[1], Christa Seipelt[2], Juliane Hellhammer[1]

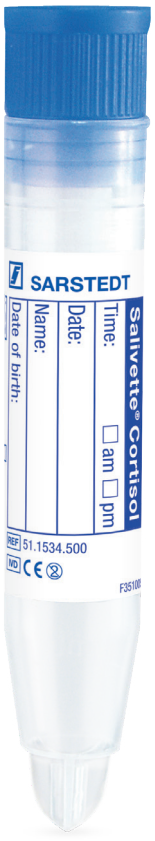
[1] Daacro GmbH & Co. KG, Max-Planck-Straße 22, 54296 Trier, Germany
[2] SARSTEDT AG & Co. KG, Sarstedtstrasse 1, 51588 Nümbrecht, Germany

Introduction

The use of saliva as a non-invasive and easy to collect bioanalytic specimen is still increasing in health care and scientific studies of stress and immunity [1,2]. Saliva can be collected under many circumstances from humans - including infants and elderly - and even animals without the main difficulty of blood collection, which is the invasiveness of the puncture resulting in increased release of stress hormones [2]. Saliva is commonly collected with two methods: through passive drool, which is still considered as the gold standard, and with a swab, similar as it is been used by dentists. The Salivette® swab collection device provides a hygienic and easy to handle method to collect saliva samples. It was the first device specially developed for easy saliva collection over 30 years ago. The easy-to-follow instructions for use of the Salivette® lead to increased participant compliance resulting in better and stronger data sets. The participant places the swab in the mouth where it easily absorbs saliva. The saturated swab is then placed back into the Salivette® container, capped and sent to the laboratory. The Salivette® Cortisol, REF 51.1534.500 code blue with a synthetic swab, is specially designed and evaluated for cortisol determination. Due to its high clinical relevance, cortisol is one of the most frequent analytes that can be determined from saliva.

In autumn 2018, a bulletin was released stating that the use of SARSTEDT swabs may introduce bias in salivary analyte results [3]. This is contradictory to a recent publication saying that cortisol levels in saliva were unaffected by using the Salivettes system [4]. Based on this inconsistency, a comparative study was initiated and then performed by the *Saliva Lab Trier*.

Therefore, to estimate possible influences of the swab-based collection using the Salivette® Cortisol, the present study was performed, comparing cortisol levels in native saliva samples (collected through unstimulated passive drool = reference samples) with cortisol levels after pipetting whole saliva of the same sample onto the swab of the Salivette® Cortisol.



Study design

The study population consisted of 32 participants (22 females, 10 males; demographic characteristics can be found in Table 1). Volunteers were asked to collect 4 saliva samples at different times of the day (at awakening, 30 and 45 minutes after awaking and at 8 pm) to cover diverse levels of cortisol and to use the Cortisol Awakening Response (CAR), the pronounced increase in cortisol release within the first 30 to 45 minutes after awakening [5], as a standardized and well-researched biomarker.

	Age [years]	Height [cm]	Weight [kg]	BMI [kg/m^2]
Mean	45.3	173.0	73.5	24.4
SD	17.4	11.3	17.3	4.8
Minimum	9	137.0	27.0	14.4
Maximum	86	190.0	115.0	37.1
N	32	32	32	32

Table 1. Demographic characteristics of the study population

To preclude that cortisol levels were affected by eating or tooth brushing, participants refrain from eating and drinking (except for water) 1 h prior to saliva collection; tooth brushing was not allowed during the post-awakening period. Furthermore, participants were asked to rinse their mouth with water 10 minutes before each saliva collection (except for the first morning sample). Participants collected unstimulated whole saliva in the polypropylene tube of the Salivette® (without the swab and the tube insert) and stored the samples in the refrigerator until the next day, when they handed the 4 samples to the lab. In the lab, samples were vortexed and 500 µl of saliva was pipetted onto the swab of the Salivette® Cortisol. After 5 min soaking, the whole Salivette® Cortisol, including the insert tube and the swab, and the native sample were frozen at -20 °C using a temperature control system until further analysis. The longest time period between freezing and day of analysis was 24 days; details can be found in Table 2.

Days	24	20	19	14	12	10	7	6	5	3	1	Σ
Sample sets	2	2	1	5	4	4	3	4	3	1	3	32

Table 2. Storage period of the collected samples at -20 °C.

On day of assay, samples were thawed and centrifuged at 1500 x g for 15 minutes to remove mucins and other particulate matter. All samples of the study were assayed on the same day and all the samples of one participant (4 x native + 4 x swab samples) were analyzed on the same plate to avoid any bias due to inter-assay variation.

Salivary cortisol levels were determined at the *Saliva Lab Trier* using a high sensitivity salivary cortisol competitive immunoassay kit manufactured by Salimetrics LLC (Carlsbad, USA). The kit contains a 96-well microplate coated with monoclonal antibodies to cortisol. All samples were analyzed in duplicate. The assay was carried out according to the manufacturer's instructions. After assay completion, optical density was read on a BioTek ELx808 microplate reader at 450 nm (with correction at 490 nm). The calculations for determining the cortisol concentration on each sample were carried out following the kit manufacturer's instructions and using the Gen 5 v.3.08 software which enabled, as required, a 4-parameter non-linear regression standard curve to be generated. Analyses were performed according to the quality and safety guidelines of the laboratory. Assay quality was measured by calculating the intra-assay coefficient of variation (CV). Samples that showed a CV higher than 10% were reanalyzed unless the cortisol concentration was very low in which case a higher CV % was expected. Inter-assay variability was checked with the low and high controls on each microplate. The intra- and interassay coefficients of variation were 2.35 % (2.31 % native; 2.38 swab) and 3.44 %, respectively.

Salimetrics report the lower limit of sensitivity (analytical sensitivity) as the minimal concentration of cortisol that can be distinguished from 0 as 0.193 nmol/L. The functional sensitivity was determined by assaying 60 samples at a concentration level resulting in a CV of approximately 20%. The functional sensitivity of the salivary cortisol ELISA is 0.773 nmol/L.

Results

Salivary cortisol is expected to increase in response to the impulse of awakening, reaching its peak within 30 to 45 minutes after awakening and decreasing within the day, resulting in low evening values. The overall mean values of the native (not processed) and swab samples (saliva pipetted onto the swab of the Salivette® Cortisol), showed the expected pattern and no great differences between the two collection methods (Table 3, Figure 1).

	Awakening [nmol/L]	+30 min [nmol/L]	+45 min [nmol/L]	8 pm [nmol/L]
Mean Native	9.70	16.34	16.20	2.03
SD	4.91	6.21	7.57	1.16
Mean Swab	9.81	15.97	15.58	2.42
SD	5.00	6.30	7.27	1.13

Table 3. Measures of salivary cortisol in native and swab samples. The cortisol concentration is given in nmol/L. The cortisol values of N = 32 participants were averaged for each time point

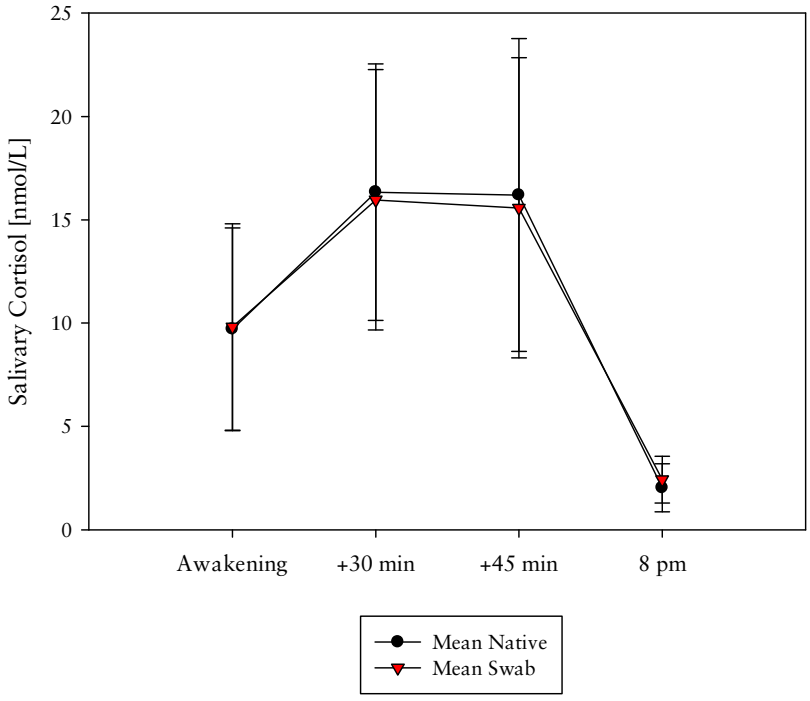


Figure 1. Mean salivary cortisol levels in native and swab samples

The swab samples measure a little lower in samples of median cortisol values (around 16 nmol/L; + 30 and + 45 minutes samples) and a bit higher in samples of very low cortisol levels (around 2 nmol/L; evening samples at 8 pm). Therefore, the salivary cortisol levels separated for each time point and each participant is given in Figure 2.

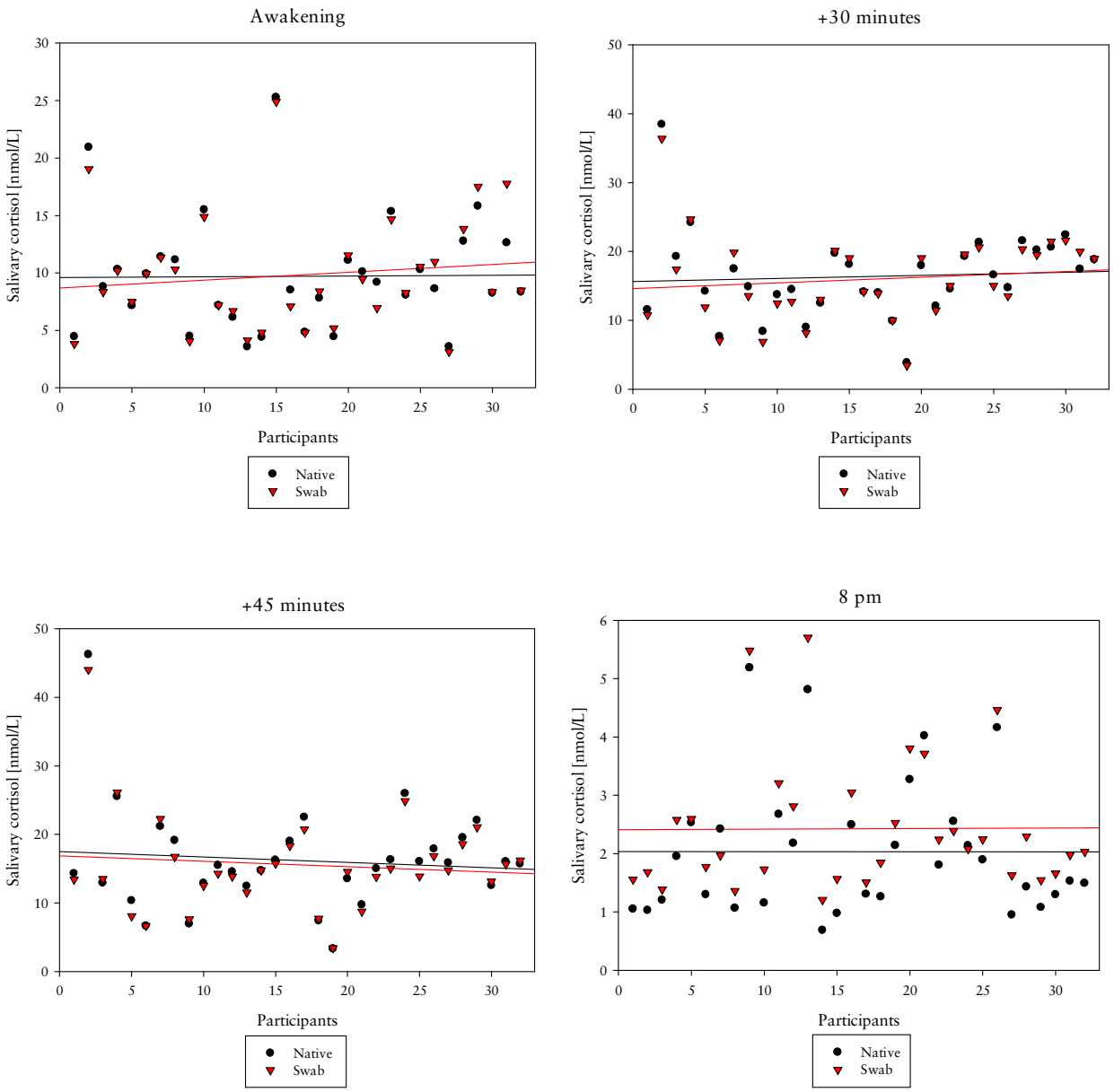


Figure 2. Salivary cortisol levels in native and swab samples, separated for each time point

To evaluate a possible correlation between the both methods, a scatterplot was created (Figure 3 and 4). The plot indicates nearly perfect correlation between native and swab samples with only a few outliers.

The correlation coefficient is 0.991 ($p<0.001$), indicating almost no differences between the two collection methods.

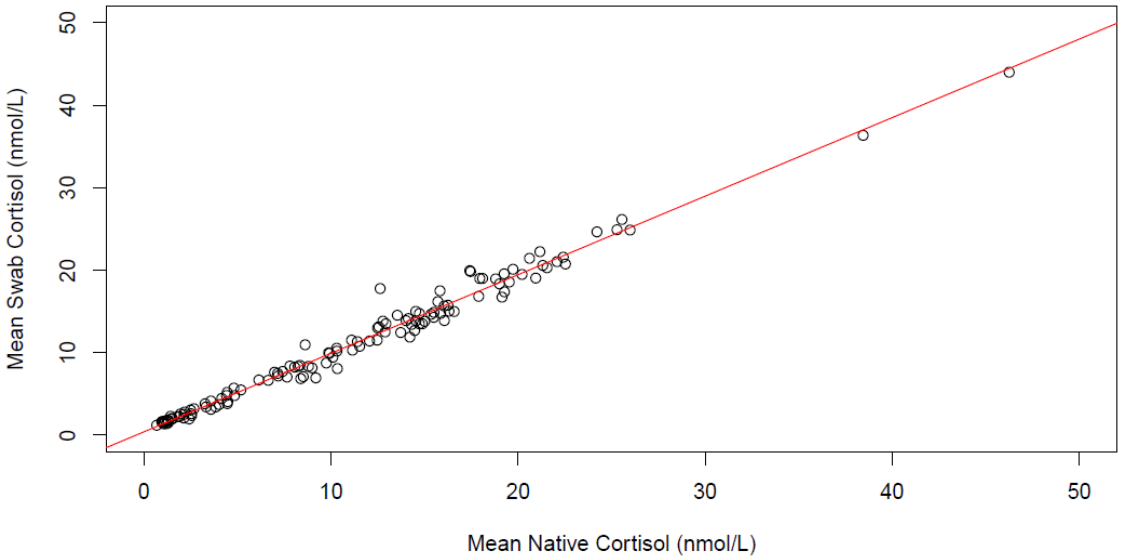


Figure 3. Scatterplot of native cortisol versus swab cortisol levels (mean values of the two assay duplicate measurements)

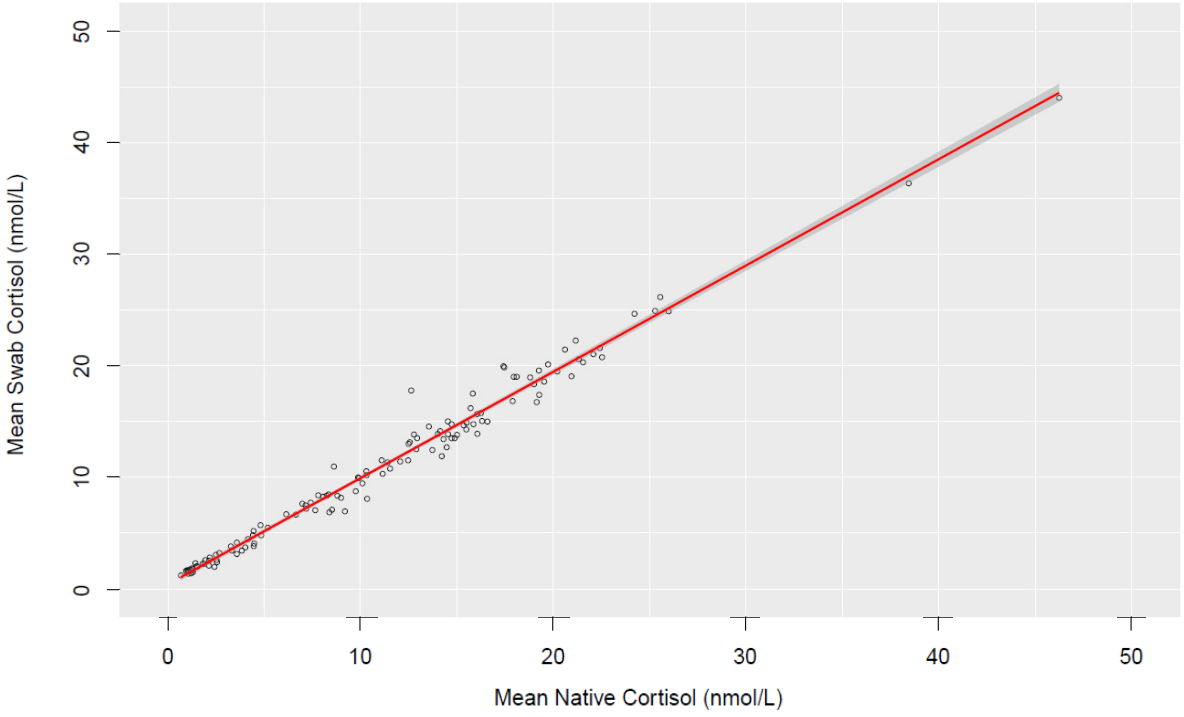


Figure 4. Scatterplot with the 95% confidence interval (gray shading)

The R-squared with 0.983 is in the same range of the correlation coefficient. The calculated standard deviation shows an expected deviation from the mean of 1.012 (residual standard error).

The regression model therefore has a high goodness of fit. A linear relationship between the two variables can be assumed.

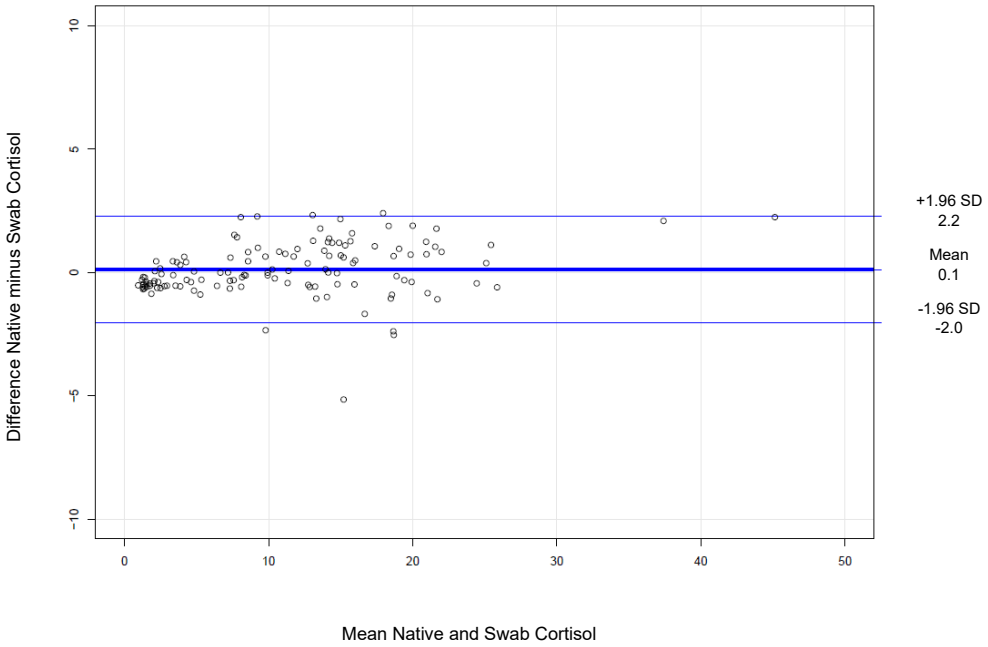


Figure 5. Bland-Altman plot, the blue lines indicate the average difference ± 1.96 SD

The Bland-Altman plot (Figure 5) shows the difference between the measured values of both methods (Y-axis) and their mean value (X-axis). It displays a high degree of agreement between the two cortisol measuring methods, since almost all values lie within the standard deviation of ±1.96,

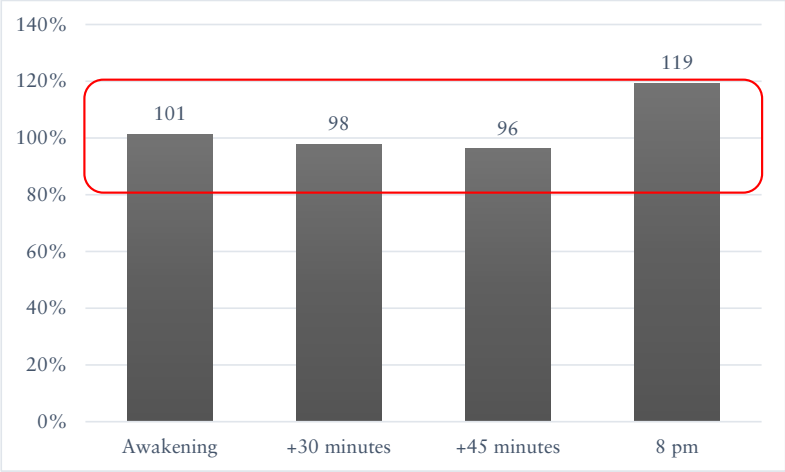


Figure 6. Mean salivary cortisol levels of swab samples for each time point relatively to the native samples (set to 100 %). The red band represents the acceptable range (±20% acceptance criterion)

Besides the correlation of the two different method, also the specificity of the method is important and should be considered. Figure 6 shows the values of the mean swab samples relatively to the mean native samples (set to 100 %) for each time point.

All four mean values are within the acceptance range of ±20 %, again showing that the most variation using the Salivettes® occur at low cortisol values (around 2 nmol/L; evening samples at 8 pm).

Conclusions

The main advantage of measuring biomarkers in saliva is the non-invasive and easy sample collection procedure. It is important to use a swab method that does not change the biomarker concentration and closely matches passive drool results.

In the present study, the concentration of cortisol in saliva samples collected with the passive drool method was compared with the cortisol concentration of the same samples after pipetting onto the swab of the Salivette® Cortisol. No significant differences between the two collection methods can be oberserved. A nearly perfect correlation ($r=0.911$, $p<0.001$) with only a few outliers indicates very low bias between the two collection methods. It could be demonstrated that sampling with the Salivette® Cortisol is a good alternative to the passive drool method ensuring the preservation of sample integrity.

Although discussed vividly in the literature, a bias in salivary cortisol results using the Salivette® Cortisol cannot be confirmed. The present study indicates that the results from the Salivette® Cortisol very closely match passive drool levels. Evaluating the minimal difference between both methods against the benefit of using the Salivette® Cortisol, data sets surely benefit from a better compliance of individuals using the Salivette® Cortisol.

This study was performed in August 2020 by Contract Research Organization DAACRO (Trier, Germany) and its *Saliva Lab Trier*. As an ISO 9001:2015 certified unit with a special focus on stress related research, daacro's *Saliva Lab Trier* commits itself to work with and provide only collections devices and analytical methods that are evaluated and scientifically established. The lab functions as an excellence laboratory for Salimetrics, USA and serves as a consultant for academic and industrial research projects. Further, the lab develops customer tailed saliva diagnostic devices for individuals and health care providers.

References

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SARSTEDT AG & Co. KG
P.O. Box 12 20
D-51582 Nümbrecht
Phone: +49 2293 305 0
Fax: +49 2293 305 3992
export@sarstedt.com
www.sarstedt.com