



101 Innovation Blvd., Suite 302  
State College, PA 16803 USA  
(T) 814-234-7748 (F) 814-234-1608  
800-790-2258 (USA & Canada only)  
[www.salimetrics.com](http://www.salimetrics.com)  
[techservices@salimetrics.com](mailto:techservices@salimetrics.com)

## SALIVARY $\alpha$ -AMYLASE ASSAY KIT

Catalog No. 1-1902, 96-Well Kit

For Research Use

### Intended Use

Salimetrics Salivary  $\alpha$ -Amylase Assay Kit is specifically designed for the kinetic measurement of salivary  $\alpha$ -amylase activity. It is not intended for use with serum/plasma or for diagnostic use. It is intended only for research use with saliva. Please read the complete kit insert before performing this assay. For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

### Introduction

Technical advances that make the assessment of biomarkers in saliva possible have enabled researchers to non-invasively study biosocial processes related to stress in naturalistic contexts. Much of the attention has focused on the activity of the limbic hypothalamic-pituitary-adrenal (LHPA) axis as indexed by individual differences and intra-individual change in salivary cortisol. Recently, it was suggested that the nearly exclusive focus of this endeavor on salivary cortisol may not enable researchers to adequately operationalize the psychobiology of the stress response (1). Physiologists have known for decades that the stress response has at least two principal components. One involves corticotropin-release hormone, activation of the LHPA axis, and the secretion of glucocorticoids (e.g., cortisol) into circulation. The second involves activation of the locus coeruleus/autonomic (sympathetic) nervous system and the release of catecholamines (e.g., norepinephrine) into the blood stream (2). Theorists argue that to advance our understanding of how biological, social, and behavior processes interact to determine risk versus resilience the next generation of studies will need to employ analytical models that operationalize both the behavioral and biological sides of the equations using multi-method and trait measurement approaches (1). Unfortunately, our ability to do so has been restricted because in contrast to the highly sensitive, accurate, and valid measurement of LHPA products in saliva (i.e., cortisol, dehydroepiandrosterone), the non-invasive measurement of autonomic (sympathetic) nervous system activity in saliva (i.e., catecholamines) has been problematic (3).

In an attempt to overcome this problem, we conducted an extensive computerized literature search for potential surrogate markers of autonomic (sympathetic) nervous system activation that could be measured accurately in saliva. A substantial literature reveals that salivary  $\alpha$ -amylase is a correlate of sympathetic activity under conditions of stress. Studies show that levels of salivary  $\alpha$ -amylase increase under a variety of physically (i.e., exercise, heat and cold) and psychologically (i.e., written examinations) stressful conditions (4) in human subjects. Early studies showed that salivary  $\alpha$ -amylase concentrations are predictive of plasma catecholamine levels, particularly norepinephrine (NE), and are highly correlated with NE changes in response to stress (4). However, more recent studies call this relationship into question (5,6). The literature does show that stress-related increases in salivary  $\alpha$ -amylase can be inhibited by the adrenergic blocker propranolol (7,8) and also that beta-adrenergic agonists are capable of stimulating  $\alpha$ -amylase release without increasing salivary flow (9, 10). This link suggests that the same stimuli that increase autonomic (sympathetic) arousal may activate sympathetic input to the salivary glands. Interestingly, several studies showed that cortisol levels did not in any way correlate with  $\alpha$ -amylase during stress (1,4, 5), suggesting that individual differences in  $\alpha$ -amylase represent a response to a stress signal independent of the LHPA axis. Studies show salivary  $\alpha$ -amylase measurements may be employed as a non-invasive measure of autonomic (sympathetic nervous system activation and are related to a variety of behavioral, social, health, and cognitive phenomena in human subjects (11-15).

### Test Principle

This method utilizes a chromagenic substrate, 2-chloro-p-nitrophenol linked with maltotriose. (16) The enzymatic action of  $\alpha$ -amylase on this substrate yields 2-chloro-p-nitrophenol, which can be spectrophotometrically measured at 405 nm. The amount of  $\alpha$ -amylase activity present in the sample is directly proportional to the increase in absorbance at 405 nm. For ease of use, the reaction is read in a 96-well microtiter plate with controls provided.

### Precautions

1. Do not mix components from different lots of kits.
2. When using a multichannel pipette,  $\alpha$ -amylase substrate solution should be added to duplicate wells at the same time, using the dispensing mode to avoid introducing bubbles into the wells.
3. See 'Material Safety Data' at the end of procedure.
4. Routine calibration of pipettes is critical for the best possible assay performance and accurate timing is critical for correct assay results.
5. Cigarette use can be associated with lower alpha-amylase scores returned by this assay because acid aldehydes in cigarette smoke are capable of changing the function and/or structure of the alpha-amylase enzyme.
6. Caffeine and other exogenous substances with sympathomimetic properties may be associated with higher alpha-amylase levels.
7. Time since eating, and the use of alpha-amylase inhibitors are also likely to be associated with alpha-amylase levels.

**Storage** All components of this kit are stable at 2 - 8°C until the kit's expiration date.

### Reagents

1.  **$\alpha$ -Amylase Substrate:** 45 mL of a ready-to-use liquid preparation of 2-chloro-p-nitrophenol linked with maltotriose. Sodium azide, at 0.01%, is added as a preservative. Reagent warming trough is also provided.
2.  **$\alpha$ -Amylase Controls:** One vial containing 100  $\mu$ L of a high level of salivary  $\alpha$ -amylase activity and one vial containing 100  $\mu$ L of a low level of salivary  $\alpha$ -amylase activity in a saliva-like matrix. See vials for ranges. Controls come pre-diluted. **Do not dilute.**
3.  **$\alpha$ -Amylase Diluent:** 30 mL of a phosphate buffered solution containing a non-mercury preservative.

**Note:** Controls should be assayed once on each day of testing. Volume is sufficient for testing on four different days

### Materials Supplied

1. 96-well microtiter plate
2. Reagent warming trough

### Materials Needed But Not Supplied

- Precision pipette to deliver\* 8  $\mu$ L
- Precision multichannel pipette to deliver\* 320  $\mu$ L
- Vortex
- Plate reader with a 405 nm filter
- Computer software for data reduction
- Microcentrifuge tubes for sample dilutions
- Pipette tips
- Timer
- Microtiter plate 37°C incubator/rotator (Needed for heating of substrate. We do **not** recommend heating the substrate in a 37°C incubator not specifically designed for microtitre plates)

\*without employing "blow-out" mechanism

### Specimen Collection

The preferred saliva collection method is to use plain (non-citric acid) cotton Salivettes, P/N 5001. Samples may also be collected using Sorbettes, P/N 5029 (for infants) or cotton ropes, P/N 5016 or by passive drool. For accurate results collection devices should be completely saturated before removal. **Do not** add sodium azide to saliva samples as a preservative. Samples visibly contaminated with blood should be recollected. Freeze at -20°C or lower for long-term storage. Contact the technical service team at Salimetrics for more detailed information on specimen collection.

Saliva samples should be frozen prior to assay to precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Pipette clear sample into appropriate wells. Particulate matter may interfere with the reaction, leading to inaccurate results.

## Procedure

Bring all reagents to room temperature. It is recommended that samples be processed one strip at a time.

**Step 1:** Determine your plate layout. Here is a suggested layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ctrl H	S - 7										
B	Ctrl L	S - 8										
C	S - 1	S - 9										
D	S - 2	S - 10										
E	S - 3	S - 11										
F	S - 4	S - 12										
G	S - 5	S - 13										
H	S - 6	S - 14										

**Step 2:** Keep the desired number of strips in the strip holder and return the remaining strips back to the bag.

**Step 3:** Set your plate reader to incubate at 37°C, and to read in center measurement kinetic mode initially at one minute, then again two minutes later. Choose the 405 nm filter with no reference filter. For plate readers without these options, incubation can take place in a plate incubator/rotator with manual movement of the plate into and out of the plate reader for the 1 minute and 3 minute readings. Kit validation was performed under these conditions.

**Step 4:** Saliva samples are to be diluted with the  $\alpha$ -amylase diluent provided. Prepare a 1:10 dilution of the saliva by pipetting 10  $\mu$ L of saliva into 90  $\mu$ L  $\alpha$ -amylase diluent. Mix well. Further dilute by pipetting 10  $\mu$ L of the 1:10 dilution into 190  $\mu$ L  $\alpha$ -amylase diluent (1:20). Final dilution is 1:200. The 1:10 dilution will not be used and can be set aside or discarded.

**Step 5:** Heat the  $\alpha$ -amylase substrate solution to 37°C in the trough provided, using a preheated microtiter plate incubator. Be sure reagent is thoroughly warmed and mixed before use. (A minimum warm-up time of 20 minutes, from room temperature, is recommended.) **NOTE:** *We do not recommend heating the substrate in a 37°C incubator not specifically designed for microtitre plates.*

**Step 6:** For accurate timing, test only one strip at a time. Add 8  $\mu$ L of controls (prediluted) and/or diluted saliva samples to individual wells. We strongly recommend reverse pipetting to avoid introducing any bubbles into the well.

**Step 7:** Add 320  $\mu$ L of preheated (37°C)  $\alpha$ -amylase substrate solution to each well simultaneously using a multichannel pipette. Discard pipet tips to avoid reagent contamination. Do not return any of the  $\alpha$ -amylase substrate solution to the bulk tray once you have dispensed it into the wells. This could contaminate the bulk tray contents and affect any subsequent testing. Any well containing bubbles at the time of reading must be repeated.

**Step 8:** If reading kinetically in 37°C plate reader, immediately place plate in reader and start reader. Otherwise, follow these steps:

Start timer **immediately** and mix (500-600 RPM) at 37°C.

Read OD at **exactly** 1 minute and return to mixing at 37°C. **Save** 1 minute OD readings.

Read OD again at **exactly** 3 minutes. **Save** 3 minute OD readings.

**Step 9 (all methods):** Subtract the one minute readings from the three minute reading and multiply by the conversion factor (see below). The conversion factor takes the 1:200 sample dilution into account for the prediluted controls and samples.

It is convenient to set up an Excel spread sheet to subtract the OD's and multiply by the conversion factor. Results are expressed in U/mL.

## Limitations

Samples that exceed 400 U/mL (linearity limit) should be diluted by 400. Results should be multiplied by 2. Values too low to read at a 1:200 dilution can be rerun at a dilution of 1:100. Results should be divided by 2.

## Calibration

This procedure is standardized using the millimolar absorptivity of 2-chloro-p-nitrophenol under the test conditions described.

## Calculations

$$\frac{\Delta\text{Abs./min} \times \text{TV} \times \text{DF}}{\text{MMA} \times \text{SV} \times \text{LP}} = \text{U/mL of } \alpha\text{-amylase activity in sample}$$

Where:  $\Delta\text{Abs./min}$  = Absorbance difference per minute  
TV = Total assay volume (0.328 mL)  
DF = Dilution factor  
MMA = Millimolar absorptivity of 2-chloro-p-nitrophenol (12.9)  
SV = Sample volume (0.008 mL)  
LP = Light path = 0.97 (specific to plate received with kit)

$$\frac{\Delta\text{Abs./2} \times 0.328 \times 200}{12.9 \times 0.008 \times 0.97} = \Delta\text{Abs.} \times 328^* = \text{U/mL } \alpha\text{-amylase activity}$$

Example: If change in absorbance was 0.3, then  $0.3 \times 328 = 98.4$  U/mL

\*If using a Tecan plate reader and data capture by Assayzap software, multiply by 0.0328.

NOTE: Multiply value by 0.01667 to convert to SI Units (nKat/L)

## Expected Values\*

Adult range, (n=75) mean = 92.4 U/mL

Absolute range = 3.1 - 423.1 U/mL

\*To be used as a guide only. Each laboratory should establish its own range.

## $\alpha$ -Amylase Assay Performance Characteristics

### A. Recovery:

Known quantities of amylase were added to five saliva samples containing different levels of endogenous amylase.

Sample	Endogenous (U/mL)	Added (U/mL)	Expected (U/mL)	Observed (U/mL)	Recovery (%)
1	72.18	65.19	137.37	134.73	98.1
2	123.97	77.09	201.06	224.43	111.6
3	103.28	10.01	113.29	10.37	96.5
4	29.99	6.72	36.71	40.96	111.6
5	42.01	3.14	45.15	39.44	87.4

### B. Precision:

The intra-assay precision was determined from the mean of 10 replicates each.

Sample	N	Mean (U/mL)	Standard Deviation (U/mL)	Coefficient of Variation (%)
H	10	474.6	11.8	2.5
M	10	108.8	7.2	6.7
L	10	17.7	1.3	7.2

The inter-assay precision was determined from high and low  $\alpha$ -amylase samples run in eight individual runs.

Sample	N	Mean (U/mL)	Standard Deviation (U/mL)	Coefficient of Variation (%)
H	8	166.0	6.0	3.6
L	8	10.6	0.6	5.8

### C. Linearity of Dilution:

Two saliva samples were diluted with  $\alpha$ -amylase diluent and assayed.

Sample	Dilution Factor	Expected (U/mL)	Observed (U/mL)	Recovery (%)
1			40.96	
	1:2	20.48	19.35	94.5
	1:4	10.24	9.76	95.3
	1:8	5.12	4.57	89.3
2	1:16	2.56	2.13	83.2
			1943.16	
	1:3	647.72	649.21	100.2
	1:9	215.91	224.64	104.0
	1:27	71.97	78.08	108.5

### D. Sensitivity:

The lower limit of sensitivity is governed by the change in absorbance. A change in absorbance less than 0.01 will not result in a reliable value. Samples should be rerun at a higher concentration.

## Material Safety Data\*

### Hazardous Ingredients

The substrate reagent contains potassium thiocyanate. *Poisonous*. Do not ingest.

The substrate reagent contains 0.01% sodium azide as a preservative. Do not ingest. Explosive metal azides may form in copper or lead plumbing. Disposal requires large volumes of water to prevent the build up of azide.

We recommend the procedures listed below for all kit reagents.

### Handling

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard absorbent materials while wearing protective clothing. Follow local regulations for disposal.

### Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing, give oxygen and call a physician.

\*The above information is believed to be accurate but is not all-inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from contact with reagents.

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## Seller's Limited Warranty

"Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller's satisfaction to be defective. All claims should be submitted in written form. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability, in all cases, will be limited to the purchased cost of the kit.

**It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller's product or out of the breach of any express or implied warranties."**