17-alpha Hydroxy Progesterone ELISA (Saliva)

For the quantitative determination of 17-hydroxyprogesterone in saliva

For "*In Vitro* Diagnostic" use within the United States of America.
This product is for "Research Use Only" outside of the United States of America.

Catalog Number: 20-17OHU-E01-SLV
Size: 96 wells
Version: 9.0 2018/10 – ALPCO 2.0

1. Introduction

1.1. Intended Use

An enzyme immunoassay for the quantitative *in vitro diagnostic* measurement of active free 17-hydroxyprogesterone in saliva. Measurements of 17-hydroxyprogesterone are used as an aid in the diagnosis of various disorders of the adrenal glands or the ovaries, and as an aid in the diagnosis of late onset of 21-hydroxylase deficiency, a common cause of Congenital Adrenal Hyperplasia.

This test is not intended for newborn screening.

1.2 Summary and Explanation

The steroid 17-α-Hydroxyprogesterone (17-α-OHP) is produced by both the adrenal cortex and gonads. Even though 17-α-OHP has relatively little progestational activity, it is of intense clinical interest because it is the immediate precursor to 11-desoxycortisol (Cpd-S). Because Cpd-S is produced by 21-hydroxylation of 17-α-OHP, measurement of 17-α-OHP is a useful indirect indicator of 21-hydroxylase activity. In congenital 21-hydroxylase deficiency, the most common variety of Congenital Adrenal Hyperplasia (CAH), 17-α-OHP is secreted in abundant excess. It is moderately elevated in the 11-β-hydroxylase deficiency as well. Measurement of 17-α-OHP is therefore valuable in the initial diagnosis of CAH.

Adult non-pregnant women:

In adult non-pregnant women in the childbearing age group, 17-α-OHP concentrations vary over the menstrual cycle with luteal phase concentrations being higher than follicular phase concentrations. This is because 17-α-OHP is secreted parallel with progesterone from maturing follicles or from the corpus luteum. There is also a diurnal variation of 17-α-OHP concentrations. This rhythm is parallel with adrenal cortisol secretion such that maximum 17-α-OHP concentrations are measured in samples obtained in the morning.

Congenital adrenal hyperplasia:

The principal application of the 17-α-OHP is in the diagnosis of CAH in newborns with ambiguous genitalia and in virilized adolescent girls. Since 17-α-OHP is the immediate precursor to 11-desoxycortisol, basal 17-α-OHP concentrations are sharply elevated in patients with 21-hydroxylase deficiency and to a lesser degree in patients with 11-hydroxylase deficiency. Because 17-α-OHP concentrations are so markedly elevated in newborns and adolescent girls afflicted with CAH, a single basal measurement is all that is normally required to make the diagnosis.

Late onset adrenal hyperplasia:

More recently, 17-α-OHP concentrations have been utilized in the evaluation of androgenized women where late onset 21-hydroxylase is suspected. This condition is clinically very subtle and since the presentation is the same as classical polycystic ovarian disease, basal plasma 17-α-OHP concentrations, unlike classical congenital adrenal hyperplasia, are normal. The diagnosis is made by administration of an ACTH stimulation test.

2. PRINCIPLE OF THE TEST

The Salivary 17-α-OHP ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with a polyclonal antibody (rabbit) directed towards an antigenic site on the 17-α-OHP molecule. Endogenous 17-α-OHP of a patient sample competes with a 17-α-OHP-horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of 17-α-OHP in the sample. After addition of the substrate solution, the intensity of color developed is inversely proportional to the concentration of 17-α-OHP in the patient sample.

3. Warnings and Precautions

1. This kit is for in vitro diagnostic use only. For professional use only.
2. **This test is NOT intended for screening newborn babies.**

3. All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
4. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
5. The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch and used in the frame provided.
6. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
7. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur.
8. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
9. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
10. Allow the reagents to reach room temperature (21-26°C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
11. Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
12. Do not smoke, eat, drink, or apply cosmetics in areas where specimens or kit reagents are handled.
13. Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
14. Handling should be done in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
15. Do not use reagents beyond expiry date as shown on the kit labels.
16. All indicated volumes must be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiter plate readers.
17. Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
18. Avoid contact with *Stop Solution* containing 0.5 M H_2SO_4. It may cause skin irritation and burns.
19. Some reagents contain Proclin 300, BND*, and MIT* as preservatives. In case of contact with eyes or skin, flush immediately with water.
20. TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
21. Chemicals and prepared or used reagents must be treated as hazardous waste according to the national biohazard safety guideline or regulation.
22. For information on hazardous substances included in the kit please refer to Material Safety Data Sheets. Material Safety Data Sheets for this product are available upon request.

*BND = 5-bromo-5-nitro-1,3-dioxane ; MIT = 2-methyl-2H-isothiazol-3-one

4. Reagents

4.1. Reagents provided

1. ***Microtiter wells***, 12x8 (break apart) strips, 96 wells; Wells coated with a polyclonal anti-17α-OHP antibody
2. ***Standard (Standard 0-5)*****,** 6 vials, 1 mL, ready to use.
Concentrations: 0, 10, 50, 250, 500, 1000 pg/mL
Conversion: pg/mL x 3.03 = pmol/L
Contain non-mercury preservative.

3. ***Control Low and High***, 2 vials, 1.0 mL each, ready to use.
Refer to vial label or QC-Datasheet for control values and ranges
Contain non-mercury preservative.

4. ***Enzyme Conjugate***, 1 vial, 26 mL, ready to use.
17-α-OHP conjugated to horseradish Peroxidase.
Contain non-mercury preservative.
5. ***Substrate Solution***, 1 vial, 25 mL, ready to use; Tetramethylbenzidine (TMB).
6. ***Stop Solution***, 1 vial, 14 mL, ready to use; contains 0.5M H_2SO_4.
Avoid contact with the stop solution. It may cause skin irritations and burns.
7. ***Wash Solution***, 1 vial, 30 mL (40X concentrated); see "Reagent Preparation".

Note: Additional *Standard 0* for sample dilution is available upon request.

4.2 Material required but not provided

- A microtiter plate calibrated reader (450 ± 10 nm).
- Calibrated variable precision micropipettes (25 µL, 100 µL, 200 µL, 250 µL).
- Absorbent paper.
- Distilled or deionized water
- Timer (60 min. range).
- Semi-logarithmic graph paper or software for data reduction

4.3 Storage Conditions

When stored at 2°C-8°C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date. Opened reagents must be stored at 2°C-8°C. Microtiter wells must be stored at 2°C to 8°C. Once the foil bag has been opened, care should be taken to close it tightly again. Opened kits retain activity for six weeks if stored as described above.

4.4 Reagent Preparation

Bring all reagents and required number of strips to room temperature prior to use.

Wash Solution

Add deionized water to the 40X concentrated Wash Solution.
Dilute 30 mL of concentrated *Wash Solution* with 1170 mL deionized water to a final volume of 1200 mL.
The 1X working Wash Solution is stable for 2 weeks at room temperature.

4.5 Disposal of the Kit

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Material Safety Data Sheet.

5. SPECIMEN Collection and Preparation

Eating, drinking, chewing gum, or brushing teeth should be avoided for 30 minutes before sampling. Otherwise, it is recommended to rinse mouth thoroughly with cold water 5 minutes prior to sampling. Do not collect samples when oral diseases, inflammation or lesions exist (blood contamination). In case of visible blood contamination, the patient should discard the sample, rinse the sampling device with water, wait for 10 minutes and take a new sample.
Note: Samples containing sodium azide should <u>not</u> be used in the assay.

5.1 Specimen Collection

It is recommended to collect saliva samples with commercially available equipment
Do not use any cotton swab for sampling, such as Salivettes; this in most cases will result in significant interferences.
To avoid arbitrary results, due to the episodic secretion pattern of steroid hormones, timing of sampling should be carefully considered. We recommend that 5 samples always be taken within a period of 2 – 3 hours (*multiple sampling*), preferably before a meal. As food might contain significant amounts of steroid hormones, it is preferable to take samples while fasting. If fasting should be a problem, the collection period should be timed just before lunch or before dinner.

5.2 Specimen Storage and Preparation

Fresh saliva samples
Immediately after arrival in the lab, fresh saliva samples should be frozen at least overnight at –20 °C.
Each saliva sample must be frozen, thawed, and centrifuged at least once to separate the mucins by centrifugation.
Storage: Immediately at –20 °C for up to 9 months.

Then samples must be thawed and centrifuged for 5 to 10 minutes at 10,000 *g*. Thereafter, the clear supernatant must be transferred into a fresh tube. Only this clear supernatant can be used as a sample for the ELISA.

The supernatant should be frozen only once. Thawed supernatant should be inverted several times prior to testing.

If a set of multiple samples must be tested, the lab must mix the aliquots of the 5 single samples in a separate sampling device and perform the testing from this mixture.

5.3 Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with *Standard 0* and re-assayed as described in the Assay Procedure.
For the calculation of the concentrations this dilution factor must be taken into account.
Example:
a) Dilution 1:10: 10 µl saliva + 90 µl *Standard 0* (mix thoroughly)
b) Dilution 1:100: 10 µl of dilution a + 90 µl *Standard 0* (mix thoroughly).

6. Assay procedure

6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control, or sample to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule, the enzymatic reaction is linearly proportional to time and temperature.

6.2 Test Procedure

Each run must include a standard curve.
All standards, samples, and controls should be run **in duplicate. All standards, samples, and controls should be run concurrently so that all conditions of testing are the same.**

1. Secure the desired number of microtiter strips in the holder.
2. Dispense **25 µL** of each 17α-OHP *Standard, Control,* and sample (supernatant) with new disposable tips into appropriate wells.
3. Dispense **250 µL** *Enzyme Conjugate* into each well.
   Mix the plate thoroughly for 10 seconds. It is important to have a complete mixing in this step.
4. **Incubate for 60 minutes at room temperature**
5. Briskly shake out the contents of the wells.
   Rinse the wells **3 times** with 1X working Wash Solution (400 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note: The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
6. Add **200 µL** of *Substrate Solution* to each well.
7. Incubate for **15 minutes** at room temperature.
8. Stop the enzymatic reaction by adding **100 µL** of *Stop Solution* to each well.
9. Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader. It is recommended that the wells be read **within 10 minutes** after addition of the *Stop Solution*.

6.3 Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls, and samples.
2. Using semi-logarithmic graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard must be further diluted and retested or reported as > 1000 pg/mL. For the calculation of the concentrations, the dilution factor must be taken into account for any additional dilutions used for retested samples.

6.3.1 Example of Typical Standard Curve

The following data is for demonstration only and **cannot** be used in place of data generations at the time of assay.

Standard	Optical Units (450 nm)
Standard 0 (0 pg/mL)	1.92
Standard 1 (10 pg/mL)	1.72
Standard 2 (50 pg/mL)	1.47
Standard 3 (250 pg/mL)	0.91
Standard 4 (500 pg/mL)	0.64
Standard 5 (1000 pg/mL)	0.41

7. Expected Normal values

In order to determine the normal range of salivary 17α -OHP, two studies were performed using saliva samples from 129 apparently healthy children ages 6 – 12 years, 132 males ages 21 to 70 years, and 252 non-pregnant women with regular menstrual cycles, ages 21 to 50 years. Saliva samples were collected in the morning, frozen at -20˚C, and analyzed using this ELISA kit. The following ranges were calculated from this study.

Summary Normal ranges for Salivary 17α-OHP ELISA

	Age group		Mean	S.D.	Range 5 - 95%
Children	6 – 12 years	N = 129	16.9 pg/mL	9.5 pg/mL	3.0 – 32.9 pg/mL
Women	21 – 50 years	Follicular phase: N = 124	22.0 pg/mL	11.1 pg/mL	8.2 – 41.1 pg/mL
		Luteal phase: N = 128	51.2 pg/mL	17.3 pg/mL	28.1 – 84.8 pg/mL
Men	21 – 70 years	N = 152	24.9 pg/mL	12.6 pg/mL	10.6 – 54.8 pg/mL

The results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests. Since 17a-OHP levels show diurnal cycles, it is recommended that the samples be obtained the same hour each day. Furthermore, it is recommended that each laboratory determine its own range for the population tested.

8. Quality Control

Good laboratory practice requires that controls be run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day-to-day validity of results. Use controls at both normal and pathological levels. The controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values and ranges stated on the QC sheet always refer to the current kit lot and should be used for direct comparison of the results. It is also recommended to make use of national or international Quality Assessment programs to ensure the accuracy of the results. Employ appropriate statistical methods for analyzing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid. In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods. After checking the above-mentioned items without finding any error contact ALPCO.

9. Performance Characteristics

9.1 Assay Dynamic Range

The range of the assay is between 3.6 – 1000 pg/mL.

9.2 Specificity of Antibodies (Cross Reactivity)

The following materials have been evaluated for cross reactivity. The percentage indicates cross reactivity at 50% displacement compared to 17α-OHP.

Steroid	% Cross-reactivity
17-α-OH Progesterone	100%
Estriol	< 0.01
Estradiol 17β	< 0.01
Testosterone	< 0.01
Dihydrotestosterone	< 0.01
DOC	0.05
11-Desoxycortisol	1.40
Progesterone	1.20
DHEA	< 0.01
DHEA-S	< 0.001
Cortisol	< 0.01
Corticosterone	< 0.05
Aldosterone	< 0.01
Androstenedione	< 0.01
Dehydroepiandrosten sulfate	< 0.01
Prednisone	< 0.01

9.3 Sensitivity

The analytical sensitivity of the ELISA was calculated by subtracting 2 standard deviations from the mean of 20 replicate analyses of the Zero Standard (S0).

Analytical sensitivity is: 2.5 pg/mL

The Limit of Blank (LoB) is 2.0 pg/mL
The Limit of Detection (LoD) is 3.6 pg/mL
The Limit of Quantification (LoQ) is 11.3 pg/mL

9.4 Reproducibility

9.4.1 Intra-Assay

The intra-assay reproducibility was determined by replicate measurements of 6 saliva samples using this ELISA kit. The within assay precision is shown below:

	sample 1	sample 2	sample 3	sample 4	Sample 5	sample 6
Mean (pg/mL)	24.2	37.5	63.3	90.3	447.4	820.4
S.D.	1.9	1.4	4.6	4.5	17.8	29.0
CV (%)	8.0	3.6	7.3	5.0	4.0	3.5
n =	20	20	20	20	20	20

9.4.2 Inter-Assay

The inter-assay variation was determined by duplicate measurements of six saliva samples on 10 different days using this ELISA kit. Mean of the duplicate measurements is given below.

	sample 1	sample 2	sample 3	sample 4	sample 5	Sample 6
Mean (pg/mL)	9.4	50.7	101.1	211.8	512.5	800.2
SD	1.0	1.7	7.6	8.3	10.3	38.2
CV (%)	10.4	3.4	7.5	3.9	2.0	4.8
n =	20	20	20	20	20	20

9.4.3 Inter-Lot

The inter-assay (between-lots) variation was determined by repeated measurements of six saliva samples in three different kit lots.

	sample 1	sample 2	sample 3	sample 4	sample 5	Sample 6
Mean pg/ml	10.2	44.3	100.4	205.3	515.7	798.1
SD pg/ml	1.3	1.5	6.1	3.6	19.5	46.8
CV (%)	10.4	3.1	5.5	1.5	1.3	6.4
n =	9	9	9	9	9	9

9.5 Recovery

Recovery of the Salivary 17-α-OHP ELISA was determined by adding increasing amounts of the analyte up to 500 pg/mL to three different saliva samples containing different amounts of endogenous analyte. Each sample (non-spiked and spiked) was assayed and analyte concentrations of the samples were calculated from the standard curve. The percentage recoveries were determined by comparing expected and measured values of the samples.

Sample		**1**	**2**	**3**	**4**	**5**	**6**
Concentration pg/mL		3.7	17.9	282.8	824.1	1050	1200
Average % Recovery		101.7	102.1	104.1	98.5	99.3	100.4
Range %Recovery	from	97.7	96.6	98.2	93.1	93.6	94.5
	to	103.9	107.5	109.5	103.8	103.6	105.1

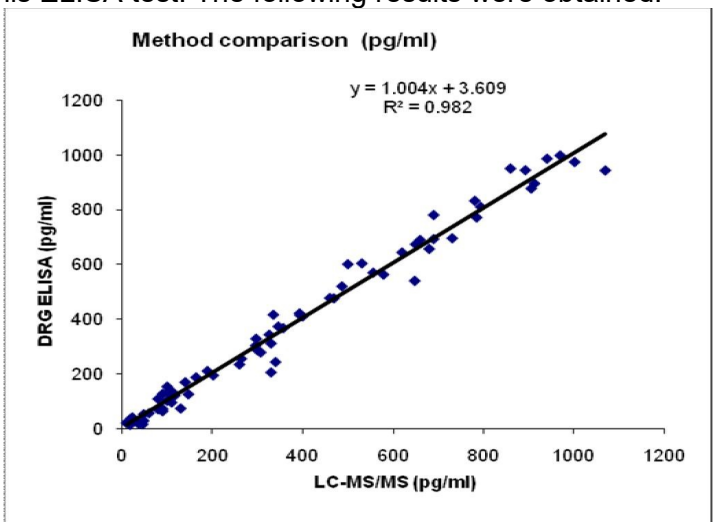
9.6 Linearity

Six saliva samples containing different amounts of analyte (spiked and unspiked) were serially diluted with zero standard and assayed with the Salivary 17-α-OHP ELISA. Three native samples were serially diluted up to 1:128, and 3 samples were spiked with 17α-OHP and then serially diluted up to 1:10. The percentage recovery was calculated by comparing the expected and measured values for 17α-OHP. An assay linearity of 3.6 – 1000 pg/mL has been identified as the usable range. Samples above this range must be diluted and re-run.

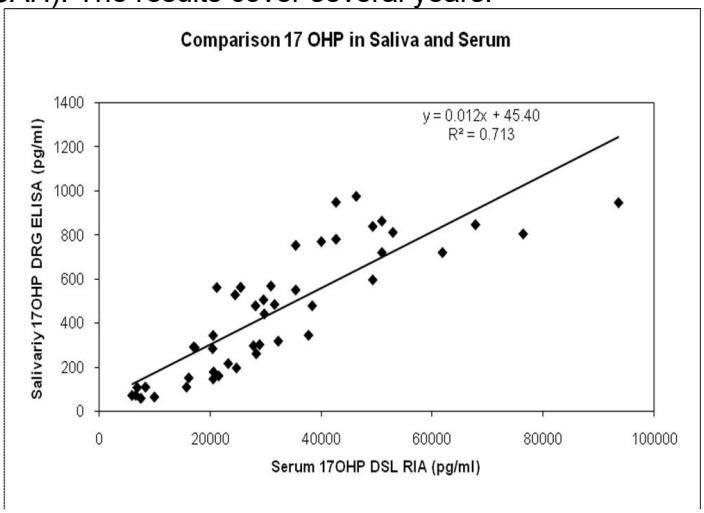
Sample		**1**	**2**	**3**	**4**	**5**	**6**
Concentration pg/mL		39.3	96.6	194.9	816.6	1050.0	1200.0
Average % Recovery		99.3	95.0	100.5	97.6	99.3	100.4
Range %Recovery	from	87.2	86.1	91.5	93.8	93.6	94.5
	to	107.4	99.0	113.2	103.5	103.6	105.1

9.7 Comparison Studies

A study was performed using non spiked Saliva from adult normal and CAH-patients were assayed in parallel with this ELISA and a reference LC-MS. Seventy-six patient saliva samples were tested on both the LC-MS and this ELISA test. The following results were obtained.



Another study was performed using 60 serum and saliva samples taken in parallel at 8:00 am and assayed using the this ELISA for the saliva samples and a Coated Tube RIA for 17-OHP for the sera samples. The patients were a collective of 22 children and young adults suffering from congenital adrenal hyperplasia (CAH). The results cover several years.



Salivary 17-α-OHP ELISA(ng/mL)

10. Limitations of Use

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice. Any improper handling of samples or modification of this test might influence the results.
The patient should not eat, drink, chew gum or brush teeth for 30 minutes before sampling. Otherwise rinse mouth thoroughly with cold water 5 min prior to sample collection. Do not collect samples when oral diseases, inflammation or lesions exist (blood contamination).

10.1 Interfering Substances
Blood contamination of more than 0.04% in saliva samples will affect results, and usually can be seen by eye. Therefore, samples containing any visible blood should not be used. Concentrations of sodium azide > 0.02% interferes in this assay and may lead to false results.

10.2 High-Dose-Hook Effect
No hook effect was observed in this test.

11. Legal Aspects
11.1 Reliability of Results
The test must be performed exactly as per the manufacturer's instructions for use. Moreover, the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, enough controls for validating the accuracy and precision of the test. The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact ALPCO.

11.2 Therapeutic Consequences
Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 11.1. Any laboratory result is only a part of the total clinical picture of a patient. Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived. The test result itself should never be the sole determinant for deriving any therapeutic consequences.

11.3 Liability
Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement. Claims submitted due to customer misinterpretation of laboratory results subject to point 11.2. are also invalid.
Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

12. REFERENCES

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