SALIVARY PROGESTERONE EIA KIT

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I. Intended Use and Description

The Eagle Biosciences Salivary Progesterone EIA Kit is designed and validated for the quantitative measurement of progesterone in human saliva.

II. Assay Background

Progesterone (4-pregnen-3, 20-dione) is one of the 21-carbon steroids secreted by the corpus luteum of the ovary in females during the normal menstrual cycle. It is also produced in low concentrations by the adrenal cortex in both males and females. In pregnancy the placenta is a major source of progesterone after the seventh gestational week.

Progesterone is synthesized from cholesterol. Of all the biologically active steroids in man progesterone is the most closely related to cholesterol and transformation of cholesterol into progesterone involves only a few biosynthetic steps (1).

Most of the progesterone in the circulation is bound to carrier proteins. Approximately 79% is bound to albumin and about 18% to cortisol binding globulin (CBG). Only a small fraction of progesterone – about 2.5% in non-pregnant women of fertile age – occurs as free hormone. It has been proposed that only the free hormone fraction is metabolically active (2,3). In saliva the majority of progesterone occurs in the free form and enters the saliva via intracellular mechanisms and reflects the free form in serum(4).

Progesterone has two main biological functions. First, it transforms the estrogen stimulated endometrium into the secretory phase, which allows implantation of the fertilized ovum. Secondly, it sustains the pregnancy by decreasing uterine contractility (5, 6). During the follicular phase progesterone concentrations are low prior to the mid-cycle gonadotropin surge (7, 8, 9, 10). Immediately after the LH surge concentrations begin to rise rapidly and reach maximum levels at the middle of the luteal phase. Circulating levels of progesterone together with estradiol have been used to evaluate luteal function in patients with menstrual disorders and infertility (11,12).

After conception the progesterone concentrations fluctuate at the midluteal levels for the first 5 – 6 gestational weeks. The luteo-placental shift occurs around the seventh week, after which the progesterone levels show a sustained rise, reaching peak levels 3-6 weeks before term (13, 14, 15). At term the levels have decreased by 20-30% of their peak level.

Measurement of maternal progesterone level is useful for confirming the diagnosis of ectopic pregnancy. Progesterone values in ectopic pregnancies are significantly lower than in normal intrauterine pregnancies (16,17,18). Measurements of the maternal progesterone concentrations have also been suggested for the clinical assessment of threatening abortion, hydatidiform mole and rhesus isoimmunisation (12, 17).

III. Assay Principle

The Salivary Progesterone EIA kit is based on the competition principal and microplate separation. Progesterone calibrators and unknown amounts of progesterone in saliva samples compete with a fixed amount of progesterone conjugated to horse radish peroxidase (Progesterone-HRP) for binding sites with a rabbit progesterone monoclonal antiserum bound to GARGG (goat anti-rabbit gamma globulin) coated wells of a microplate. After incubation, unbound components are washed away, enzyme substrate solution is added and a blue color formed. This reaction is stopped with an acid solution to produce a yellow color. The optical density is then read at 450 nm. The amount of Progesterone-HRP detected is inversely proportional to the amount of progesterone in a sample.

IV. Reagents Supplied and Reagent Preparation

Store all other reagents at 2 to 8°C. Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers. Expiration dates and lot numbers are printed on the labels.

1. GARGG Plate: One 96 well microplate (12x8 breakable strip wells) coated with goat anti-rabbit gamma globulin placed in a resealable foil bag with desiccant. One (1) 96 well kit is sufficient for 39 duplicate patient measurements.
2. **Concentrated Stock Progesterone (synthetic) solution** at a concentration of **100 ng/ml (100,000 pg/ml):** 1 bottle, 150 ul.
   Determine the amount of **working progesterone calibrators** needed and prepare based on this **example**:

Working Progesterone Calibrator 1000 pg/mL preparation:

Calibrator Concentration to prepare (pg/mL)	Stock Progesterone Concentrate to use (pg/mL)	Volume to Use (mL)	Assay Buffer to use (mL)	Final Volume (mL)
1000	100,000	0.020	1.980	2.000

Working Progesterone Calibrators 300 – 10 pg/mL preparation:

Calibrator Concentration to prepare (pg/mL)	Calibrator Concentration to use (pg/mL)	Volume to use (mL)	Assay buffer to use (mL)	Final Volume (mL)
300	1000	0.600	1.400	2.000
100	300	0.667	1.333	2.000
50	100	1.000	1.000	2.000
25	50	1.000	1.000	2.000
10	25	0.800	1.200	2.000
0			2.000	2.000

3. Assay buffer: 1 bottle, 20 ml.
4. **Stock Progesterone (synthetic) Control Concentrate 50 ng/ml (50,000 pg/ml)**: 1 bottle, 0.150 ml. Concentration is on the label and is traceable to U.S. Pharmacopeia (USP). Determine the amount of **working controls** needed and prepare based on this example:

Working Progesterone Control # 2 (500 pg/mL) preparation:

Control Concentration to prepare (pg/mL)	Stock Concentration to use (pg/mL)	Volume to use (mL)	Assay Buffer to use (mL)	Final Volume (mL)
500	50,000	0.020	1.980	2.000

Working Progesterone Control #1 (25 pg/mL) preparation:

Control Concentration to prepare (pg/mL)	Progesterone Control #2 Concentration to use (pg/mL)	Volume to use (mL)	Assay Buffer to use (mL)	Final Volume (mL)
25	500	0.050	0.950	1.000

Immediately after use, store the unused portions of the **working calibrators** and the **High** and **Low Controls** at 2-8°C. Discard if not used within 28 days of mixing.

5. **Salivary Progesterone EIA rabbit monoclonal Antibody**: 1 bottle, 6 ml. The solution is blue.
6. **Salivary Progesterone-Horseradish Peroxidase (HRP) concentrate.:** 1 amber bottle, 0.100 ml. Progesterone derivative is conjugated to horseradish peroxidase. The solution is yellow and light sensitive.
7. **Progesterone-Horseradish Peroxidase (HRP) conjugate buffer, pH 7.4**: 1 bottle, 3 ml. Use only for the preparation of the **Progesterone-HRP working reagent only**.
   Progesterone-HRP working reagent preparation: Determine the amount of **working Progesterone-HRP** needed and dilute 1:40 with conjugate buffer pH 7.4 (#7). For example, mix 0.0625 ml of **Progesterone-HRP concentrate** (#6) plus 2.437 ml with **conjugate buffer,** (#7). This is sufficient for 100 EIA wells. Immediately after use, store the unused portion of the **Progesterone-HRP working reagent** at 2-8°C. Discard if not used within 28 days of mixing.
8. Wash solution (10X concentrated) EIA #1: 1 bottle, 50 ml of phosphate buffered saline, pH 7.4. Prior to use dilute 1:10 with deionized water.
9. Color Development Reagent EIA #1: 1 amber plastic bottle, 15 ml of Tetramethylbenzidine (TMB) plus hydrogen peroxide. Light sensitive.
10. Stopping Solution EIA #1: 1 bottle of a 15 ml mixture of diluted sulfuric and hydrochloric acid solution.

*Concentration of progesterone calibrators and controls are actual and traceable to US Pharmacopeia (USP) Cat. No. 56800 Lot I1J239

V. Storage and Stability

1. When stored at 2° - 8°C, unopened reagents will retain activity until the expiration date. Do not use reagents beyond this date.
2. Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers.
3. Opened reagents must be stored at 2° - 8°C.
4. Microtiter wells must be stored at 2° - 8°C. Once the foil bag has been opened, care should be taken to reseal tightly.
5. Opened kits retain activity for 28 days if stored as described above.
6. Expiration dates and lot numbers are printed on the labels.

VI. Materials Needed But Not Provided

1. Device to dispense very accurately 50 ul of saliva.
2. Multichannel pipettors.
3. Microplate or orbital shaker
4. Vortex Mixer
5. Microplate washer (not required, plates can be washed manually).

6. Microplate reader capable of reading 450 nm with 4 parameter data reduction or comparable software.
7. Plate Sealers
8. Suitable sample collection device

VII. Sample Collection Processing

1. This samples collection and processing procedure must be followed:
   a. **A suitable collection device** is required for the collection of saliva samples when determining progesterone concentrations with the Salivary Progesterone EIA Kit
   b. Avoid food consumption, drinking coffee or alcohol, smoking or chewing gum 15 minutes prior to sample collection.
   c. Rinse mouth thoroughly with water 15 minutes prior to collection.
   d. In the **required saliva collection device** collect a minimum of 1 mL, (Use the number 1 marked on the collection tube as a reference), of whole saliva by un-stimulated
   passive drool by allowing saliva to drip off the lower lip into the graduated collection tube or by allowing saliva to accumulate in the floor of the mouth and spitting it into the collection tube. Label the sample tube with the following information:
      i. Date and time of sample collection
      ii. Patient's name
      iii. Patient's gender
      iv. Patient's date of birth
   e. The sample(s) should be sent as soon as possible after collection to the testing site, they should remain stable under average shipping conditions, including over weekends and holidays and during hot temperatures. If the sample(s) will not be sent the day of collection, store at 2-8°C until ready to be shipped.
   f. Upon arrival of samples to the testing site, the sample(s) should be kept in the collection device to maintain its integrity and freeze (≤ -15°C or below) until day of assay. On day of assay, thaw samples to facilitate precipitation of mucins. Centrifuge at 1500g for ten minutes. Bring samples to room temperature and assay.

2. Sample stability

Storage	20-28°C	37°C	2-8°C	≤-15°C (7 freeze/thaw cycles)	≤-15°C (Long term)
Stability	Up to 7 days	Up to 7 days	Up to 7 days	Up to 7 days	Up to 12 months

VIII. Assay Procedure Summary Flow Sheet

Calibrator Progesterone Sample I.D. pg/ml	Calibrator, Control, Sample (ul)	HRP progesterone Working (ul) solution	Anti-Progesterone (ul)		Diluted 10X wash solution. (ul)		Color Developer (ul)		Stopping solution (ul)	
0	50	25	50	Mix. Incubate for 2 hrs. at Room Temperature, shaking.	300	Wash 3X	125	Mix. Incubate 30 min. at room temperature	125	Mix. Read at 450 nm
10	50	25	50		300		125		125	
25	50	25	50		300		125		125	
50	50	25	50		300		125		125	
100	50	25	50		300		125		125	
300	50	25	50		300		125		125	
1000	50	25	50		300		125		125	
Control #1	50	25	50		300		125		125	
Control #2	50	25	50		300		125		125	
Sample	50	25	50		300		125		125	

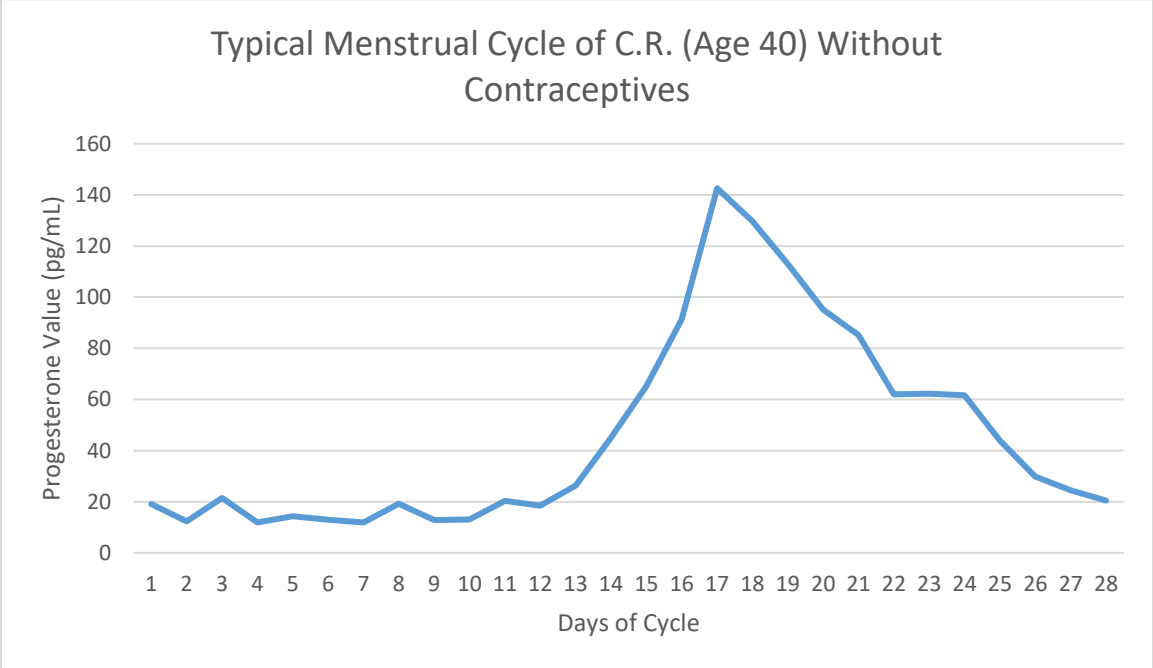
IX. Assay Procedure

1. The calibrators, controls and samples should be tested in duplicate and the mean value used to report the results.
2. To the GARGG microplate dispense **50ul** of **working Salivary Progesterone EIA calibrators** (**0, 10, 25, 50, 100, 300,** and **1000** pg/ml), **controls**, and **saliva** samples.
3. Add **25 ul** of **Progesterone-HRP working reagent** to all wells.
4. Add **50 ul** of **Progesterone EIA rabbit monoclonal antibody**.
5. Cover microplate with plastic sealer. Incubate by shaking on a microplate orbital shaker set a 500-900 rpm for **2 hrs.** at room temperature.
6. After incubation, decant the contents of the wells. Wash 3 times with 300 ul of **diluted wash solution.** After the 3rd wash, invert GARGG microplate on an absorbent paper and tap dry.
7. Dispense 125 ul of Color Development reagent EIA #1 into each well. Shake briefly (manual). Cover microplate with plastic sealer. Incubate for 30 minutes at room temperature.
8. Dispense 125 ul of Stopping Solution EIA #1 into each microtiter well of the GARGG plate. Shake briefly (manual). Color changes from blue to yellow.
9. Read at 450 nm on a microplate reader within 10 minutes.

Note: If samples exceed the upper end of the measuring range of 1000 pg/mL, dilute with zero calibrator and make appropriate concentration correction.

X. Typical Results

Typical Calibration Curve (Actual assay)			
Calibrators (pg/ml)	Mean Absorbance (450 nm)	% B/Bo	Value (pg/ml)
0	2.83		0
10	2.33	82	10
25	1.72	61	25
50	1.28	45	50
100	0.74	26	100
300	0.29	10	300
1000	0.13	4	1000
Control 1	1.66	59	28.6
Control 2	0.22	8	441.7
Sample 1	2.24	79	11.7
Sample 2	2.33	82	9.8
Sample 3	1.41	50	39.6



XI. Determination of Progesterone Concentration

1. Determine the concentrations of the controls and unknowns by interpolation using Software capable of logistics using a 4-parameter sigmoid minus curve fit.

Analytical measuring range (AMR)	10-1000 pg/ml

Conversion: 3.18 pg/ml to pmol/L. Multiply by 3.18 to convert pg/ml to pmol/L.

XII. Quality Control

The expected values for the controls are stated on the label of each control which are included in the kit. The results can only be accepted if the expected values are met. Follow federal, state and local guidelines for testing quality control materials.

XIII. Expected Progesterone Normal Ranges With The Salivary EIA Kit

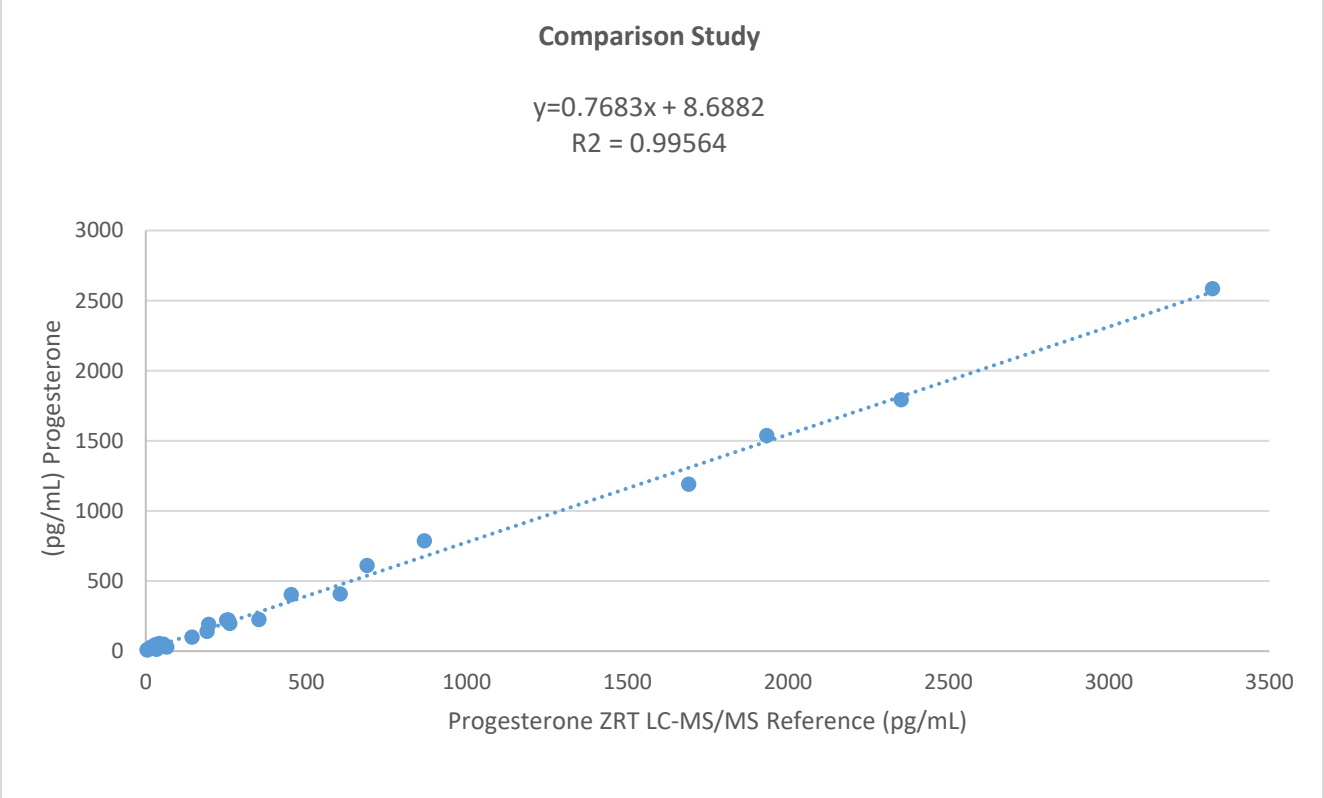
Saliva samples collected in the AM show the following values. See results below:

Progesterone pg/mL				
Female	Premenopausal n = 84	Follicular Phase	Median 19.0	Range 9.1 – 58.7
		Luteal Phase	79.0	20.4 – 219.6
	Postmenopausal n = 60	8.6		1.3 – 48.8
Male	n = 58	19.5		7.4 – 46.2

It is recommended that each laboratory establishes its own range of normal values.

XIV. Comparison Study

Twenty eight (28) saliva samples with a range of 5 – 3323 pg/mL were compared with a LC-MS/MS procedure. See results below:



XV. Performance Characteristics

A. Specificity of the Antiserum

Steroids	% Cross-reactivity
C-21 Steroids	
Progesterone	100.000
17OH-Progesterone	1.2696
Pregnenolone	0.6524
17OH-Pregnenolone	0.0036
Desoxycorticosterone	1.5584
11-Desoxycorticosterone	0.1490
Corticosterone	2.1360
Aldosterone	0.9035
Cortisol	0.2375
20α Dihydroprogesterone	0.2170
20β Dihydroprogesterone	0.1226
Pregnenolone-3-SO4	0.7519
C-19 Steroids	
Androstenedione	0.1144
Testosterone	0.1033
5 alpha DHT	0.0486
DHEA-SO4	0.0022
Androstanedione	0.0947
C-18 Steroids	
Estradiol-17 β	0.0032
Estradiol-17 α	0.0029
Estriol	0.0009
Estrone	0.0087

B. Detection limits

The Detection Limit Study for determining the limit of the blank (LoB) and Limit of detection (LoD) for the Salivary Progesterone EIA Kit was performed using several low Progesterone samples and two different reagent lot numbers that were assayed twice per day over a period of 3 days.
(Reference, CLSI EP 17-A, protocols for Determination of Limits of Detection and Limits of Quantitation).

Limit of the Blank (LoB) pg/mL	Limit of Detection (LoD) pg/mL
0.950	1.477

C. Precision and Reproducibility:

Intra-assay

The intra-assay precision was determined from the mean of 20 replicates of low, medium and high pools

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	20	24.6	1.661	6.8
Medium	20	176.5	5.365	3.0
High	20	418.8	23.186	5.5

Inter-assay

The inter-assay precision was determined from the mean average of the duplicates for 12 separated assays with low, medium and high pools.

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	12	24.2	2.4	10.0
Medium	12	178.8	7.5	4.2
High	12	432.6	28.6	6.6

Inter-lot Variation

The inter-lot precision was determined by duplicate measurements of three (3) saliva pools and three (3) spiked controls in saliva like matrix, using three (3) different reagent lots.

Saliva Samples ID	Lot # 001 mean (pg/ml)	Lot # 002 mean (pg/ml)	Lot # 003 mean (pg/ml)	Inter-lot mean (pg/ml)	Inter-lot Std. Dev. (pg/ml)	Inter-lot CV (%)
Pool 1	26.3	28.4	24.2	26.3	2.100	8.0
Pool 2	193.5	187.3	195.0	191.9	4.082	2.1
Pool 3	447.0	450.8	445.6	447.8	2.691	0.6
Control 1	21.5	24.1	22.0	22.5	1.380	6.1
Control 2	92.4	90.8	92.4	91.9	0.924	1.0
Control 3	385.2	384.1	375.7	381.7	5.196	1.4

D. **Linearity Study:**

Ten (10) sample concentrations that span the assay measuring range were prepared and assayed per EP6-A, Evaluation of the Linearity of Quantitative Measurement Procedures.

S=10 samples (dilutions)
Concentration = (C1*V1 + C10*V10)/(V1+V10)

	C1 pg/mL	V1 mL	C10 pg/mL	V10 mL	Calculated Concentration pg/mL	Observed Concentration pg/mL	Recovery %
1					8.0	8.0	100.3
2	8.0	0.889	1100.0	0.111	129.2	122.0	94.4
3	8.0	0.778	1100.0	0.222	250.4	237.4	94.8
4	8.0	0.667	1100.0	0.333	371.6	350.9	94.4
5	8.0	0.556	1100.0	0.444	492.8	448.6	91.0
6	8.0	0.444	1100.0	0.556	615.2	561.2	91.2
7	8.0	0.333	1100.0	0.667	736.4	760.6	103.3
8	8.0	0.222	1100.0	0.778	857.6	886.7	103.4
9	8.0	0.111	1100.0	0.889	978.8	1003.3	102.5
10					1100.0	1162.0	105.6

***** Targets of low and high sample concentrations.

E. Recovery

Seven (7) samples containing different levels of endogenous progesterone were spiked with known quantities of Progesterone and assayed.

Sample	Endogenous (pg/ml	Added (pg/ml)	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
1	45.6	10.0	55.6	54.7	98.3
2	16.7	50.0	66.7	64.5	96.8
3	19.9	100.0	119.9	109.3	91.1
4	11.3	500.0	511.3	501.5	98.1
5	22.6	1000.0	1022.6	1026.0	100.3
6	23.2	800.0	823.2	873.0	106.0
7	12.4	700.0	712.4	710.2	99.7

XVI. Limitations of the Procedure

1. The Salivary Progesterone EIA Kit reagents are optimized to measure progesterone in human saliva.
2. Avoid the use of samples containing blood contamination, sodium azide and thimerosal as these compounds lead to false results. Our studies indicate interference with salivary progesterone values at concentrations of 0.05% - 0.5% for these three (3) interferants tested.
3. Salivary Progesterone concentrations in pregnant women have not been established with the Salivary Progesterone EIA Kit.

XVII. Precautions

1. Only physician, clinical labs, research labs and hospital labs may acquire, possess and use the kit.
2. This kit is for research only. Follow the working instructions carefully.
3. Do not pipet reagents by mouth.
4. Do not smoke, eat or drink while performing assay.
5. Wear disposable rubber gloves.
6. Treat all saliva samples as potentially infectious.
7. Do not mix reagent lot numbers or alter in any way the reagents in this kit.
8. Avoid contact with Color Development Reagent (TMB). It contains solvents that can irritate skin and mucus membranes. If contact is made, wash thoroughly with water.
9. Avoid contact with stopping solution. It contains acid. If contact is made, rinse thoroughly with water.

XVIII. Selected and Cited References

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