CAFFEINE

ELISA

For research use only (RUO)
Not for use in diagnostic procedures

CAFN-96-U 96 tests

Version: V02
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ENGLISH

INTENDED USE

The Caffeine ELISA (CAFN-96) is intended for highly sensitive, quantitative determination of Caffeine in human saliva, plasma, serum, food extracts, drinks, water, and other biological fluids.

For research use only. Not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The Caffeine ELISA is a competitive immunoassay using a capture antibody technique. Calibrators, controls and samples containing Caffeine are incubated over night with a highly specific monoclonal anti-Caffeine antibody. During this incubation the formed antibody-Caffeine complexes are captured onto the pre-coated wells of the microplate. Then, enzyme label (Caffeine conjugated to horseradish peroxidase) is added, which competes with Caffeine present in calibrators, controls and samples for the free binding sites captured on the coated wells during a 1-hour incubation step. Unbound enzyme label is then removed by a washing step, and TMB (tetramethylbenzidine) substrate is added to the wells. In a further 30-minutes incubation step a chromophore is formed (turns from colorless to blue) in inverse proportion to the amount of Caffeine originally present in the samples, controls and calibrators. The color turns from blue to yellow after the addition of an acidic stop solution and can be measured at 450 nm.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Microtiter Plate precoated with antibody capture molecules	12x8 wells	CAFN-MP	Stored under 250 µL of protection buffer; wash 2x before use
Plate Sealer	3 pieces	-	
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 mL	B-WB2	Dilute with 900 mL of deionized water
Calibrators with preservatives	5 vials 0.5 mL	CAFN-CASET	Ready to use
Control low / high[1)] with preservatives	2 vials 0.5 mL	CAFN-CONSET	Ready to use
Dilution Buffer with preservatives	1 vial 30 mL	CAFN-DB	Ready to use
Anti-Caffeine Antibody with preservatives	1 vial 5.5 mL	CAFN-AB	Ready to use; blue color
Enzyme Label Caffeine conjugated to HRP	1 vial 3 mL	CAFN-EL	Ready to use; yellow color
TMB Substrate buffered with citrate	1 vial 11 mL	B-TMB	Ready to use
Stop Solution 0.25 M sulfuric acid (H_2SO_4)	1 vial 11 mL	B-STS	Ready to use **Irritant**

Table 1

[1)] Lot specific amount of Caffeine (see QC data sheet added to the kit).

STORAGE AND SHELF LIFE OF REAGENTS

Sealed / Unopened Reagents	
Store at 2-8°C until expiration date. Do not use past expiration date.	
Opened / Reconstituted Reagents	
Microtiter Plate	Cover unused strips with a plate sealer, return them to the aluminium/plastic pouch and reseal along the entire edge of zip-seal. Store for up to 6 months at 2-8°C
Wash Buffer diluted	Store at 2-8°C up to 6 months.
Calibrators	Store at 2-8°C until expiration date.
Controls	
Anti-Caffeine Antibody	
Enzyme Label	
Dilution Buffer	
TMB Substrate	
Stop Solution	

Table 2

MATERIALS REQUIRED BUT NOT PROVIDED

- 50 µL Precision pipette with disposable tips. Repeater or multichannel pipette for 25 µL, 50 µL and 100 µL.
- Disposable polypropylene tubes for the dilution of samples.
- 1000 mL cylinder for the dilution of the Wash Buffer Concentrate.
- Microtiter plate washer or squeeze bottle for diluted Wash Buffer.
- Blotting paper.
- Refrigerator.
- Microtiter plate orbital shaker.
- Microtiter plate reader for measurement of absorbance at 450 nm.
- Saliva Collection Devices (Salivettes with cotton roll) can be ordered with NovoLytiX (order code: **B-SVC50-U).**

PRECAUTIONS

Safety precautions

- This test is for research use only, not intended for use in diagnostic procedures and must be handled by qualified personnel, in accordance with good laboratory practices (GLP).
- TMB Substrate and Stop Solution: The Substrate Solution (B-TMB) contains Tetramethylbenzidine (TMB). The Stop Solution (B-STS) contains 0.25 M sulfuric acid. Each of those reagents is irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothes. Wear suitable protective clothing, gloves and eye protection. After contact with eyes or skin, wash immediately with plenty of water.
- Unused above solutions should be disposed of according to local state and federal regulations.

Technical precautions

Kit components

- Read this instruction for use (IFU) carefully before carrying out the test. Test performance will be adversely affected, if reagents are incorrectly diluted,

modified or stored under conditions other than those as detailed in this IFU.

- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Every effort should be made to ensure that no cross contamination occurs between reagents, samples or between wells.
- Microtiter plate wells cannot be re-used.
- Mix the reagents well before use.

Assay procedure

- It is highly recommended that Calibrators and Controls are assayed in duplicates, preferably the samples as well.
- Change disposable tips after each pipetting step.
- The measuring range of the standard curve covers 3 to 300 ng/mL. If the expected Caffeine concentrations of the samples exceed 300 ng/mL, the samples should be diluted with Dilution Buffer accordingly.

SPECIMEN COLLECTION (SALIVA)

Saliva can be collected by passive drooling, spitting or using saliva collection devices (e.g. Salivettes). The Salivettes can absorb up to 3 mL of saliva. This ELISA procedure calls for 0.1 mL of saliva.

- The use of "neutral" untreated Sarstedt Salivettes with the transparent caps (order code: 51.1534) is recommended. These Salivettes can be ordered with NovoLytiX (order code: **B-SVC50-U**).
- **The use of cotton swabs containing citric acid (i.e. Salivettes with green caps from Sarstedt) leads to wrong and irreproducible results. Do not use them at all.**
- Do not stimulate saliva flow by chewing gums or eating lemons.
- Individuals should perform the saliva collection on a day without sporting activities and any intense efforts, unless the study protocol forces to do it.
- The last meal and drinks except water must be taken at least 30 minutes before starting a saliva collection. Rinse the mouth with water 15 minutes before each collection time point.
- Caffeine-containing food and drinks should not be eaten/drunk during the entire day before the start of the collection period.
- Individuals should avoid brushing their teeth, with or without toothpaste, during sampling periods. It is likely that individuals with gingivitis will contaminate the saliva with blood leading to unknown consequences.
- On the collection day, if possible, no medicines should be taken.

SPECIMEN STORAGE AND SHIPMENT

Storage: The saliva samples absorbed in the cotton swabs may be stored in the saliva collection device for up to 7 days at 2-8°C. If not assayed within one week after collection, samples should be frozen and may be stored for at least 12 months at ≤ -20°C. Do not add biocides to the saliva samples as this may lead to false results.

Shipment: Home- or outpatient-collected saliva samples can be shipped at ambient temperatures with a duration for up to three days. Before shipment such collected saliva samples can be kept in the refrigerator at 2-8°C for several days. There is no deterioration of Caffeine, however bacterial and fungal growth may happen.

SERUM, PLASMA, FOOD EXTRACTS, DRINKS, AND OTHER BIOLOGICAL FLUIDS

The NovoLytiX Caffeine ELISA is particularly suited and validated for saliva. However, serum, plasma, food extracts, drinks, and other biological fluids can be measured with this ELISA. For the above samples, a dilution of at least 1 in 10 using Dilution Buffer (order code: CAFN-DB) is recommended.

ASSAY PROCEDURE

1. Dilute the samples with Dilution Buffer, if necessary. Saliva samples can be measured undiluted, if very low Caffeine values are expected. For all other samples, a dilution of at least 1 in 10 is highly recommended.
2. Use a plate with enough 8-well strips to test the desired number of Calibrators, Controls and samples. Remove excess strips from the holder, cover them again with the attached Plate Sealer and re-seal them in the aluminium/plastic foil pouch. Store refrigerated.

   Empty the wells and wash the strips twice using at least 300 µL of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.

3a. Pipet 100 µL of Dilution Buffer (blank) in duplicate into wells A1+A2.

3b. Pipet 50 µL of Dilution Buffer (Zero Calibrator) in duplicate into wells B1+B2.

   Pipet 50 µL of Calibrator A in duplicate into wells C1+C2.

   Pipet 50 µL of Calibrator B in duplicate into wells D1+D2.

   Pipet 50 µL of Calibrator C in duplicate into wells E1+E2.

   Pipet 50 µL of Calibrator D in duplicate into wells F1+F2.

   Pipet 50 µL of Calibrator E in duplicate into wells G1+G2.

3c. Pipet 50 µL of Low Control in duplicate into wells H1+H2.

   Pipet 50 µL of High Control in duplicate into wells A3+B3.

3d. Pipet 50 µL of each (diluted) sample into the subsequent wells.

4. Add 50 µL of Antiserum (blue solution) to each well, except wells A1+B1 (blank).
5. Cover the plate with a Plate Sealer, place it for 1 min on a plate orbital shaker set at 600 rpm and then incubate for 16-24 hours at 2-8 °C.
6. Add 25 µL of Enzyme Label (yellow solution) to all wells.

7. Cover the plate with the Plate Sealer, place it for 1 min on a plate orbital shaker set at 600 rpm and then incubate for 1 hour at 2-8 °C.

Important: *Allow the TMB Substrate to equilibrate to 18-28°C prior to use in step 9.*

8. Remove and discard the Plate Sealer. Aspirate or invert the plate to empty the solution from each well and wash five times using at least 300 µL of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
9. Add 100 µL of TMB Substrate to all wells.
10. Cover the plate to protect it from direct light, place it on a plate orbital shaker set at 600 rpm and incubate for 30 ± 2 minutes at 18-28°C.
11. Add 100 µL of Stop Solution to all wells. Place the plate for 10 seconds on a plate orbital shaker set at 600 rpm and proceed to step 12 within 15 minutes.
12. Read the absorbance at 450 nm in a microtiter plate reader.

QUALITY CONTROL

A thorough understanding of this IFU is necessary for the successful use of the product. Reliable results will be obtained only by precise laboratory techniques (current GLP guidelines) and accurately following this IFU.
Since there are no controls for Caffeine commercially available, we recommend using internal saliva or plasma/serum pools containing different levels of Caffeine as internal quality controls. The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the Controls are lot-specific and are printed on the QC data sheet delivered with each test kit.
If the performance of the assay does not meet the established limits and repetition has excluded errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices; ii) ELISA reader settings; iii) expiration dates of reagents; iv) storage and incubation conditions; v) TMB Substrate solution should be colorless before use; and vi) purity of water.

STANDARDIZATION

The Calibrators of the Caffeine ELISA are standardized with Supelco's certified reference material, Cerilliant® (Merck, order code: C-051).

RESULTS

Standard Curve

Record the absorbance at 450 nm for each calibrator and blank well. Average the duplicate values, subtract the average of the blank wells and record averages (=corrected average absorbance). Calculate the binding (B) of each pair of calibrator wells as a percent of Zero Calibrator (B_0 or Total, T), with the blank-corrected absorbance of the Zero Calibrator taken as 100 %.

$$B/B_0\ (\%) = \quad B/T\ (\%) = \frac{\text{net absorbance}}{\text{net absorbance of Zero Calibrator}} \times 100$$

Plot the percent bound (B/B_0 or B/T, vertical axis) versus the concentration of Caffeine in ng/mL (horizontal axis) using a lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four-parameter logistic (4-PL) or similar algorithm.

Samples and controls

- Record the absorbance at 450 nm for each control and each sample wells. Subtract the average of the blank wells and record the absorbance (=corrected average absorbance). Calculate, as described above, the binding of each pair of sample wells as a percent of Zero Calibrator (B_0 or T), with the blank-corrected absorbance of the Zero calibrator taken as 100%.
- Locate the B/B_0 (B/T) value of the samples on the vertical axis, draw a horizontal line intersecting the standard curve and read the Caffeine concentration (ng/mL) from the horizontal axis.
- Correct the measured Caffeine concentration of the samples by multiplying with the respective sample dilution applied.

See Table 3 and Figure 1 for examples of results and standard curve. ***Results and standard curves are for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.***

PERFORMANCE CHARACTERISTICS

Specificity: The 50% binding (cross-reactivity) of the anti-Caffeine monoclonal antibody with different compounds (serially diluted with Dilution Buffer) were tested according to the assay procedure and are presented in Table 4.

Detection Limit (Analytical Sensitivity, LoB): 0.8 ng/mL. 15 wells of Dilution Buffer (Zero Calibrator) were assayed in two independent runs. The minimum detectable concentration in 50 µL of Dilution Buffer was calculated by subtracting two standard deviations of averaged OD values from the OD of Zero Calibrator and intersecting this OD value with the standard curve obtained in the same run.

Inter-Assay Precision: 12.9 %. The inter-assay precision was calculated from the results of 5 to 11 independent runs with 3 samples within the standard range as well as with the low and high controls. The results are presented in Table 6.

Dilution Linearity/Parallelism: 99.7 %. Four saliva samples with high amounts of Caffeine were sequentially diluted with Dilution Buffer and assayed according to the assay procedure. The dilution recovery results are presented in Table 7.

Spiking Recovery: 94.8%. Two saliva samples were spiked with increasing amounts of Caffeine. One spiked sample set was diluted 1:10 with Dilution Buffer while the other was diluted 1:20 with Dilution Buffer. Both sample sets were then analyzed according to the assay procedure. The results are presented in Table 8.

Method Comparison: The comparison was done with 31 saliva samples from 4 different donors collected before and after consumption of a Caffeine pill. The samples were analyzed using this Caffeine ELISA (CAFN-96) and compared to the results obtained with a LC-MS/MS method [Lin et al. (2022), Front. Nutr. 8;787225]. The subsequent linear regression analysis resulted in a

correlation factor of R^2 = 0.950, an intercept of -1.47 ng/mL and a slope of 0.93. The correlation is presented in Figure 2.

APPENDIX I

TABLES AND FIGURES

Examples of Results

	Conc. (ng/mL)	Absorbance (OD)	B/B0 (B/T) (%)	CV Conc. (%)	Calc. Conc. (ng/mL)
Blank Blank **Avg.**		0.060 0.064 **0.062**			
Zero Calibrator Zero Calibrator **Avg.**	**0**	1.874 1.844 **1.860**	100.8 99.2 **100.0**	**0.7**	
Cal A Cal A **Avg.**	**3**	1.748 1.695 **1.722**	94.0 91.1 **92.5**	**2.2**	
Cal B Cal B **Avg.**	**10**	1.525 1.517 **1.521**	81.9 81.5 **81.7**	**0.4**	
Cal C Cal C **Avg.**	**30**	1.049 1.013 **1.029**	56.2 54.4 **55.3**	**2.3**	
Cal D Cal D **Avg.**	**100**	0.549 0.494 **0.522**	29.5 26.5 **28.0**	**7.6**	
Cal E Cal E **Avg.**	**300**	0.225 0.263 **0.244**	12.1 14.1 **13.1**	**10.9**	
Ctrl. High Ctrl. High **Avg.**		0.538 0.560 **0.549**	28.9 30.1 **29.5**	**4.5**	94.8 88.9 **91.8**
Ctrl. Low Ctrl. Low **Avg.**		1.586 1.549 **1.558**	85.2 83.2 **84.2**	**9.6**	7.4 8.5 **8.0**

ED80 = 10.2 ng/mL ED50 = 37.9 ng/mL ED20 = 160.7 ng/mL

Table 3

Example of Standard Curve (OD_{450})

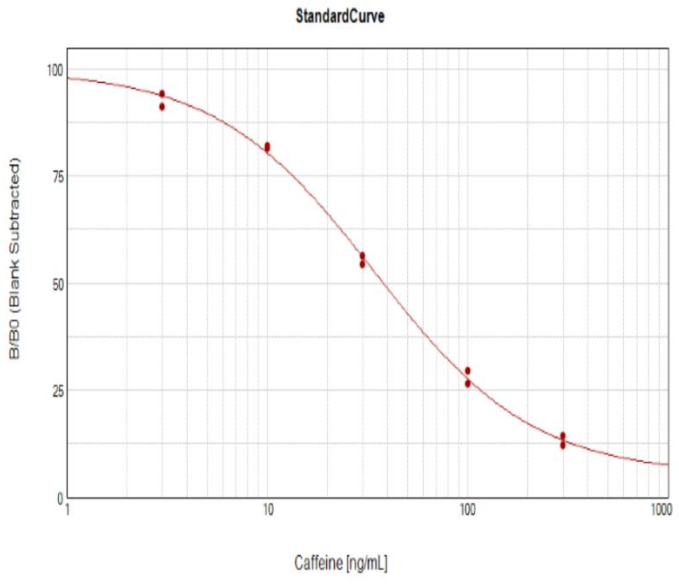


Figure 1

Specificity

Compound	Crossreactivity [%]
Caffeine (1,3,7-Trimethylxanthine)	100
Paraxanthine (1,7-Dimethylxanthine)	0.6
Theophylline (1,3-Dimethylxanthine)	28.8
Theobromine (3,7-Dimethylxanthine)	< 0.01
Xanthine	< 0.001

Table 4

Inter-Assay Precision

Sample	Mean [ng/mL]	SD [ng/mL]	CV [%]
Ctrl low	7.4	0.74	10.0
Ctrl high	88.3	10.1	11.5
C6_4_1	816.7	130.2	15.9
C6_4_2	500.2	58.0	11.6
C14_1_3	1033.6	160.9	15.6
Mean			**12.9%**

Table 6

Dilution Linearity/ Parallelism

Sample	Dilution Factor	Observed [ng/mL]	Expected [ng/mL]	Recovery O/E [%]
T1_7	1:10	169.7	164.3	103.3
	1:20	72.3	82.2	88.0
	1:40	31.8	41.1	77.4
	1:80	15.4	20.5	75.0
	1:160	11.7	10.3	113.9
	1:320	5.1	5.1	99.3
	1:640	1.9	2.6	74.0
T1_8	1:20	181.8	174.0	104.5
	1:40	72.0	87.0	82.8
	1:80	34.1	43.5	78.4
	1:160	23.5	21.8	108.0
	1:320	11.0	10.9	101.1
	1:640	6.7	5.4	123.2
	1:1280	2.7	2.7	99.3
C5_4	1:10	106.9	110.1	97.1
	1:20	62.8	55.1	114.1
	1:40	31.5	27.5	114.4
	1:80	13.5	13.8	98.1
	1:160	6.9	6.9	100.3
C11_4	1:10	101.2	99.3	101.9
	1:20	44.8	49.7	90.1
	1:40	27.5	24.8	110.8
	1:80	11.7	12.4	94.3
	1:160	7.7	6.2	123.3
Mean				**99.7%**

Table 7

Spiking Recovery

Sample	Spiking [ng/mL]	Expected [ng/mL]	Observed [ng/mL]	Recovery O/E [%]
Cat05_1	0	--	6	--
	80	86	77	89.5
	160	166	142	85.5
	320	326	271	83.1
	640	646	590	91.3
	1280	1286	1234	96.0
	2560	2566	2295	89.4
Cat06_2	0	--	26	--
	40	66	79	119.7
	80	106	111	104.7
	160	186	199	107.0
	320	346	344	99.4
	640	666	591	88.7
	1280	1306	1197	91.7
	2560	2586	2342	90.6
Mean				**94.8%**

Table 8

Method Comparison

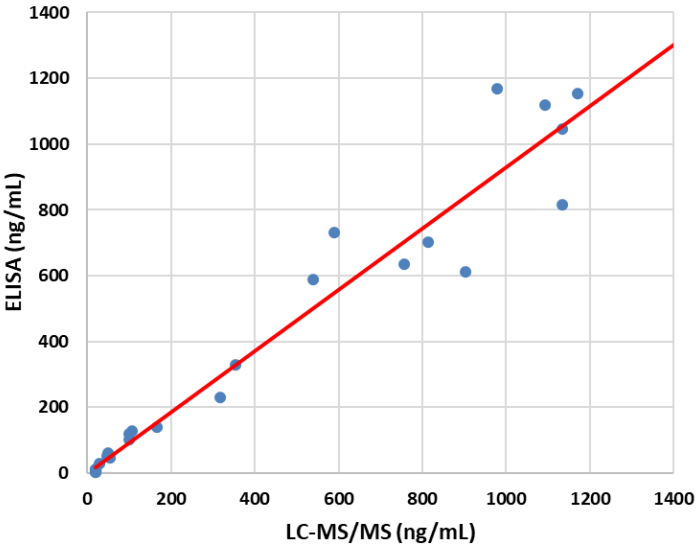
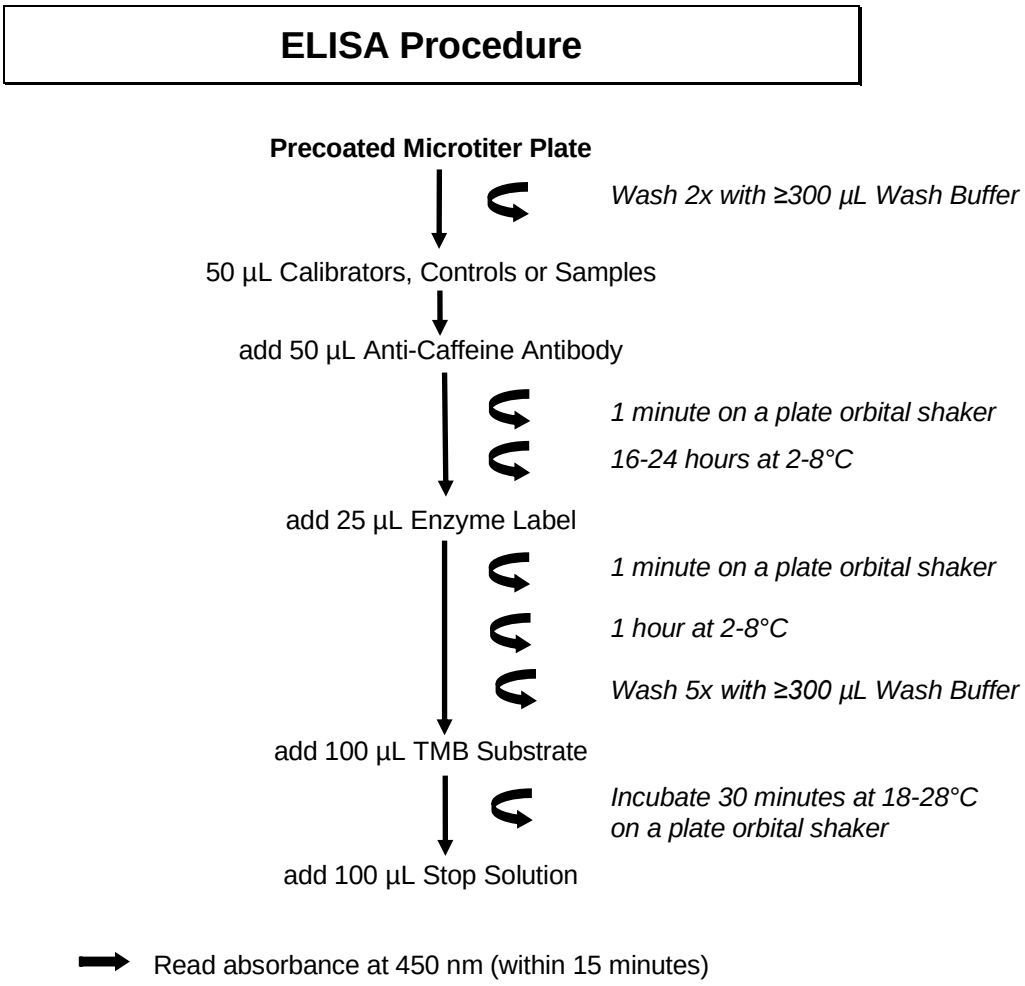


Figure 2

APPENDIX II

PIPETTING PROTOCOL



APPENDIX III

SYMBOLS

Symbol	Explanation
	Use By
REF	Order Code
LOT	Batch Code
	Contains sufficient for <n> tests
	Consult Instructions for Use
	Temperature Limitation
MP	Microtiter Plate
AB	Anti-Caffeine Antibody

Symbol	Explanation
BUF WASH 10X	Wash Buffer Concentrate (10x)
CAL A - CAL E	Calibrator A - E
CONTROL L	Control Low
CONTROL H	Control High
EL	Enzyme Label
SUBS TMB	TMB Substrate
SOLN STOP	Stop Solution
DB	Dilution Buffer

Change Log

Date	Version	Reason for change
2023-01-17	01	1st tracked version.
2023-09-25	02	Correction in Tables 3 and 7: unit is ng/mL (and not pg/mL)

Printing Date:
2023-09-25