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TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures.

HT Cyclic AMP

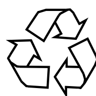
Enzyme Immunoassay Kit

Catalog # 2500-096-K

96 Well Kit

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

Trevigen, Inc.



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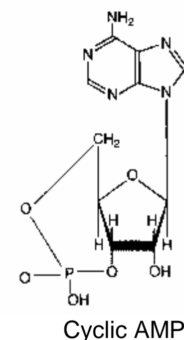
I. Description

Trevigen's HT Cyclic AMP EIA kit is a competitive immunoassay for the quantitative determination of cyclic AMP in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to cAMP to bind, in a competitive manner, the cAMP in the standards or sample or an alkaline phosphatase molecule which has cAMP covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of cAMP in either standards or samples. The measured optical density is used to calculate the concentration of cAMP. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

II. Introduction

Adenosine 3′, 5′-cyclic monophosphate (cyclic AMP; cAMP) is one of the most important "second messengers" involved as a modulator of physiological processes[3-7]. cAMP is also involved in regulating neuronal, glandular, cardiovascular, immune and other functions and actions[8-11]. A number of hormones are known to activate cAMP through the action of the enzyme adenylate cyclase which converts ATP to cAMP. These hormones include a variety of anterior pituitary peptide hormones such as corticotropin (ACTH), glucagon, calcitonin, thyroid stimulating hormone (TSH) and luteinizing hormone (LH). cAMP has been shown to be involved in the cardiovascular, nervous system, and immune mechanisms, cell growth and differentiation, and general metabolism[12-14]. There remains considerable interest in the measurement of intracellular cAMP in tissues and cell cultures, and this may help to provide an understanding of the physiology and pathology of many disease states.



Cyclic AMP

III. Precautions

FOR RESEARCH USE ONLY. ***Not*** for use in diagnostic procedures.

1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. Some solutions supplied in this kit are caustic: care should be taken with their use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (> 10 mM) such as EDTA and EGTA.
4. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
5. The cyclic AMP Standard provided, Cat# 2500-096-05, is supplied in ethanolic buffer at a pH optimized to maintain cAMP integrity. Care should be taken in handling this material because of the known and unknown effects of cAMP.

CAUTION: Some components of this kit contain chemicals that are lachrymators, corrosive and flammable. Use with caution and wear suitable protection. Refer to the Material Safety Data Sheets available on request.

IV. Materials Supplied

Catalog Number	Component	Size	Description
2500-096-P	Goat anti-Rabbit IgG Microtiter Plate	96 well plate	A plate using break-apart strips coated with goat antibody to rabbit IgG.
2500-096-01	cAMP EIA Conjugate	6 ml	A blue solution of alkaline phosphatase conjugated with cAMP.
2500-096-02	cAMP EIA Antibody	6 ml	A yellow solution of a rabbit polyclonal antibody to cAMP.
2500-096-03	Assay Buffer 2	30 ml	Sodium acetate buffer containing proteins and sodium azide as preservative.
2500-096-04	Wash Buffer Concentrate	30 ml	Tris buffered saline containing detergents.
2500-096-05	Cyclic AMP Standard	0.5 ml	A solution of 2,000 pmol/ml cAMP
2500-096-06	p-Npp Substrate	20 ml	A solution of p-nitrophenyl phosphate in buffer. Ready to use.
2500-096-07	Stop Solution	5 ml	A solution of trisodium phosphate in water
2500-096-08	Triethylamine	2 ml	Acetylation reagent
2500-096-09	Acetic Anydride	1 ml	Acetylation reagent

V. Storage

All components of this kit are stable at 4°C until the kit's expiration date.

VI. Materials Required but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µl and 1,000 µl.
3. Repeater pipets for dispensing 50 µl and 200 µl.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

VII. Sample Preparation

Trevigen's cAMP EIA is compatible with samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer 2 can be read directly from the standard curve. Please refer to the Sample Dilution recommendations on page 11 for details of suggested dilutions. However, the end user must verify that the recommended dilutions are appropriate for their sample. Samples containing rabbit IgG may interfere with the assay. Samples with very low levels of cAMP can be measured when acetylated with the provided reagents. Please refer to References 15–21 for methods for extracting cAMP from samples. Urine samples should not be used with this assay.

Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the same tissue culture media instead of Assay Buffer 2. There will be a small change in the binding associated with running the standards and samples in Media. Users should only use standard curves generated in media or buffer to calculate concentrations of cAMP. RPMI-1640 media contains high concentrations (> 350 pmol/ml) of endogenous cAMP and residual media on the cells will effect the measurement of intracellular cAMP levels.

Serum samples should be assayed immediately or frozen below −20°C. Note: EDTA plasma is not a suitable matrix for the acetylated procedure since it tends to precipitate. Tissue samples should be rapidly frozen in liquid nitrogen. Grind the frozen tissue to a fine powder under liquid nitrogen in a stainless steel mortar. Weigh the frozen tissue and homogenize in 10 volumes of cold 5% TCA in a glass-Teflon tissue grinder. Centrifuge at 600 × g for 10 minutes. Extract the supernatants with 3 volumes of water-saturated ether. Dry the aqueous extracts and run the reconstituted samples directly in the assay.

Centrifuge non-adherent cells at 250 x g for 10 minutes at 4°C. Scrape adherent cells into a 15 ml centrifuge tube and centrifuge at 250 x g for 10 minutes at 4°C. Suspend the cell pellet in 10 pellet volumes of 0.1M HCl and let sit for 10 minutes. Visually inspect an aliquot of the cells to verify cell lysis. If adequate lysis has not occurred, incubate for a further 10 minutes and inspect. Centrifuge at > 600 × g for 10 minutes. The supernatants may be assayed directly in our Direct cAMP kit, Catalog Numbers 2501-096-K, or dried down prior to reconstitution in Assay Buffer 2 and run in this kit.

VIII. Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or polypropylene tubes; avoid polystyrene.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
8. **Care must be taken to minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

IX. Reagent Preparation

1. **cAMP Standard—Non-Acetylated Version:** Allow the 2,000 pmol/ml cAMP standard solution (Cat# 2500-096-05) to warm to room temperature. Label five 12 × 75 mm glass tubes #1 through #5. Pipet 900 µl Standard Diluent (Assay Buffer 2 or tissue culture media) into tube #1 and 750 µl of Standard Diluent into tubes #2 - #5. Add 100 µl of the 2,000 pmol/ml cAMP standard to tube #1. Vortex thoroughly. Transfer 250 µl of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5. *The concentration of cAMP in tubes #1 through #5 will be 200, 50, 12.5, 3.12 and 0.78 pmol/ml, respectively. See Direct cAMP Assay Layout Sheet for dilution details. Diluted standards should be used within 60 minutes of preparation.*

2. **Acetylation Reagent:** Prepare the Acetylating Reagent by adding 0.5 ml of Acetic Anhydride (Cat# 2500-096-09) to 1 ml Triethylamine (Cat# 2500-096-08). *Use the reagent within 60 minutes of preparation.*

3. **cAMP Standard—Acetylated Version:** Allow the 2,000 pmol/ml cAMP standard solution to warm to room temperature. Label five 12 × 75 mm glass or 1.5 ml polypropylene tubes #1 through #5. Pipet 990 µl Standard Diluent (Assay Buffer 2 or tissue culture media) into tube #1 and 750 µl Standard Diluent into tubes #2 – #5. Add 10 µl of the 2,000 pmol/ml standard to tube #1. Vortex thoroughly. Transfer 250 µl of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5. The concentration of cAMP in tubes #1 through #5 will be 20, 5, 1.25, 0.312 and 0.078 pmol/ml, respectively. See Direct cAMP Assay Layout Sheet for dilution details.

   Acetylate all standards and samples by adding 10 µl of the Acetylating Reagent for each 200 µl of standard or sample. Add the reagent directly to the samples and vortex for 2 seconds immediately after the addition.

   To a 12 × 75 mm glass or 1.5 ml polypropylene tube (labeled as the Zero Standard/NSB tube) add 1 ml Standard Diluent and 50 µl of the Acetylating Reagent. Failure to acetylate the NSB and Zero standard will result in inaccurate B/Bo values. Use the acetylated standards or samples within 30 minutes of preparation.

4. **Wash Buffer:** Prepare the Wash Buffer by diluting 5 ml of the supplied concentrate (Cat# 2500-096-04) with 95 ml of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

X. Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate. If Acetylated Version of the kit is to be run, prepare the standards, samples, and Zero Standard/NSB Tube as described in Step IX.3 above.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the Ziploc bag. Store unused wells at 4 °C.

2. Pipet 100 µl of Standard Diluent (Assay Buffer 2 or Tissue Culture Media) into the NSB and the Bo (0 pmol/ml Standard) wells.

3. Pipet 100 µl of the Standards #1 through #5 into the appropriate wells.

4. Pipet 100 µl of Samples into the appropriate wells.

5. Pipet 50 µl of Assay Buffer 2 into the NSB wells.

6. Pipet 50 µl of blue Conjugate into each well except the TA and Blank wells.

7. Pipet 50 µl of yellow Antibody into each well except the Blank, TA, and NSB wells.

<u>NOTE</u>: Every well used should be ***<u>Green</u>*** in color except the NSB wells which should be ***<u>Blue</u>***. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature for 2 hours on a plate shaker at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.

9. Empty the contents of the wells and wash by adding 200 µl of wash solution to every well. Repeat the wash 2 more times for a total of *3 washes. Tap the plate between washes onto a dry lint free paper towel to remove any remaining wash buffer.*

10. Add 5 µl of the blue Conjugate to the TA wells.

11. Add 200 µl of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.

12. Add 50 µl of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

13. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

XI. Calculation of Results

Several options are available for the calculation of the concentration of cAMP in the samples. We recommend that the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic curve fitting program. If this type of data reduction software is not readily available, the concentration of cAMP can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

   Average Net OD = Average Bound OD – Average NSB OD

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

   $$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Using Logit-Log paper plot Percent Bound (B/Bo) versus concentration of cAMP for the standards. Approximate a straight line through the points. The concentration of cAMP in the unknowns can be determined by interpolation.

XII. Typical Results

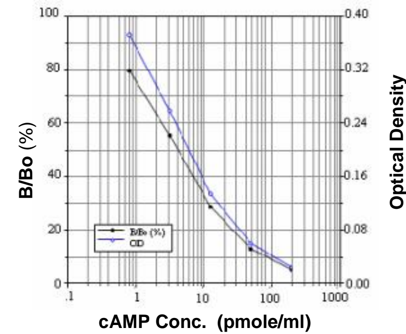
The results shown below are for illustration only and should not be used to calculate results from another assay.

	Non-Acetylated Version			Acetylated Version		
Sample	Average Net OD	Percent Bound	cAMP (pmol/ml)	Average Net OD	Percent Bound	cAMP (pmol/ml)
Blank OD	(0.085)			(0.086)		
TA	0.494			0.435		
NSB	0.000	0.03%		0.002	0.53%	
Bo	0.585	100%	0	0.403	100%	0
S1	0.029	4.9%	200	0.034	8.5%	20
S2	0.074	12.7%	50	0.087	21.6%	5
S3	0.167	28.5%	12.5	0.183	45.5%	1.25
S4	0.325	55.6%	3.125	0.296	73.4%	0.3125
S5	0.467	79.9%	0.781	0.362	89.8%	0.0781
Unknown 1	0.130	22.3%	19.39	0.061	15.1%	8.53
Unknown 2	0.398	68.0%	1.63	0.142	35.3%	2.12

XIII. Typical Standard Curves and Quality Control Parameters

Typical standard curves are shown below. These curves must not be used to calculate cAMP concentrations; each user must run a standard curve for each assay and version used.

Non-Acetylated Version



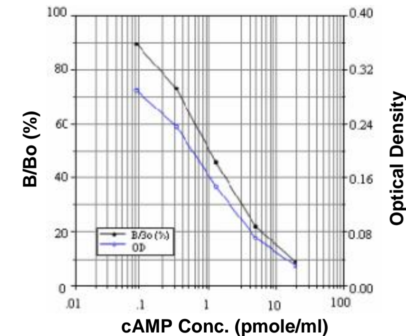
Typical Quality Control Parameters

Total Activity Added = 0.494 × 10 = 4.94

%NSB = 0.0%
%Bo/TA = 11.8%
Quality of Fit = 1.000
(Calculated from 4 parameter logistics curve fit)

20% Intercept = 23.1 pmol/ml
50% Intercept = 4.1 pmol/ml
0% Intercept = 0.8 pmol/ml

Acetylated Version



Typical Quality Control Parameters

Total Activity Added = 0.435 × 10 = 4.35

%NSB = 0.0%
%Bo/TA = 9.3%
Quality of Fit = 1.000
(Calculated from 4 parameter logistics curve fit)

20% Intercept = 5.5 pmol/ml
50% Intercept = 1.0 pmol/ml
80% Intercept = 0.2 pmol/ml

XIV. Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[22].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run with the Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #5. The detection limit was determined as the concentration of cAMP measured at two (2) standard deviations from the zero along the standard curve.

Non-Acetylated Version

Mean OD for Bo	=	0.658 ± 0.025 (3.8%)
Mean OD for Standard #5	=	0.531 ± 0.011 (2.1%)
Delta Optical Density (0–0.78 pmol/ml)	=	0.658 − 0.531 = 0.127
2 SD's of the Zero Standard	=	0.050

Sensitivity = $\frac{0.050}{0.127} \times 0.78$ pmol/ml = **0.30 pmol/ml**

Acetylated Version

Mean OD for Bo	=	0.469 ± 0.011 (2.3%)
Mean OD for Standard #5	=	0.425 ± 0.009 (2.2%)
Delta OD (0–0.078 pmol/ml)	=	0.469 − 0.425 = 0.044
2 SD's of the Zero Standard	=	0.022

Sensitivity = $\frac{0.022}{0.044} \times 0.078$ pmol/ml = **0.039 pmol/ml**

Linearity

Non-Acetylated Version

A sample containing 49.19 pmol/ml cAMP was serially diluted 5 times 1:2 in Assay Buffer 2 and measured in the assay. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration. The line obtained had a slope of 0.936 with a correlation coefficient of 0.995.

Acetylated Version

A sample containing 5.42 pmol/ml cAMP was serially diluted 5 times 1:2 in Assay Buffer 2 and measured in the Acetylated version of the assay. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration. The line obtained had a slope of 1.082 with a correlation coefficient of 0.997.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of cAMP and running these samples multiple times (n = 24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of cAMP in multiple assays (n = 8). The precision numbers listed below represent the percent coefficient of variation for the concentrations of cAMP determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Non-Acetylated Version			**Acetylated Version**		
	cAMP (pmol/ml)	Intra-assay (% CV)	Inter-assay (% CV)	cAMP (pmol/ml)	Intra-assay (% CV)	Inter-assay (% CV)
Low	1.18	10.5		0.40	7.4	
Medium	5.96	2.5		0.90	6.8	
High	18.6	2.9		5.58	7.7	
Low	1.13		13.7	4.6		11.2
Medium	4.95		11.2	0.98		11.2
High	19.18		8.4	4.75		7.9

Cross Reactivates

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer 2 at concentrations from 2,000 to 2 pmol/ml. These samples were then measured in the cAMP assay, and the measured cAMP concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
cAMP	100%
AMP	0.33%
ATP	0.12%
cGMP	< 0.001%
GMP	< 0.001%
GTP	< 0.001%
cUMP	< 0.001%
CTP	< 0.001%

XV. Sample Dilution Recommendations

Please refer to pages 4 and 5 for Sample Handling recommendations and Reagent preparation. cAMP concentrations were measured in a variety of different samples including tissue culture media, porcine serum and human saliva. cAMP was spiked into the undiluted samples which were diluted with the appropriate diluent and then assayed in the kit. Recovery values were not obtained with urine samples due to high endogenous levels of cAMP. The following results were obtained:

	Non-Acetylated Version		**Acetylated Version**	
Sample	*% Recovery*	*Recommended Dilution**	*% Recovery*	*Recommended Dilution**
Tissue Culture Media**	95.4**	None**	**	95.4**
Porcine Serum	103.3	1:2–1:10	96.2	1:2–1:10
Human Saliva	95.2	1:2–1:10	96.5	1:2–1:10
Human Urine	Do Not Use		Do Not Use	

*See Sample Preparation instructions on page 4 for details.
**However RPMI-1640 contains ~350 pmol/ml cAMP.

XVI. Assay Layout

Dilution Tables for Making Standards 1–5:

Non-Acetylated Version:

Std.	Assay Buffer 2 Vol. (µL)	Vol. Added (µL)	cAMP Conc. (pmol/mL).
1	900	100, Stock	200
2	750	250, Std. 1	50
3	750	250, Std. 2	12.5
4	750	250, Std. 3	3.12
5	750	250, Std. 4	0.78

Acetylated Version:

Std.	Assay Buffer 2 Vol. (µL)	Vol. Added (µL)	cAMP Conc. (pmol/mL)
1	990	10, Stock	20
2	750	250, Std. 1	5
3	750	250, Std. 2	1.25
4	750	250, Std. 3	0.312
5	750	250, Std. 4	0.078

Acetylation Procedure:

1. Prepare Acetylating Reagent by mixing 0.5 mL of Acetic Anhydride with 1 mL of Triethylamine.
2. Add 10 µL of the Acetylating Reagent to 200 µL of standard or sample. Vortex immediately.
3. Assay the acetylated standards and samples within 30 minutes.

Assay Protocol Flow Chart:

Well I.D.:	Blank A1, B1	TA C1, D1	NSB E1, F1	Zero Std. G1, H1	Stds. A2-B3	Samples C3-H12
Assay Buffer 2	—	—	150 µL	100 µL	—	—
Std. and/or Sample	—	—	—	—	100 µL	100 µL
Conjugate	—	—	50 µL	50 µL	50 µL	50 µL
Antibody	—	—	—	50 µL	50 µL	50 µL
Incub. 2 hours @ RT, shaking	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Asp. & Wash 3 × 200µL	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Conjugate	—	5 µL	—	—	—	—
Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Incub. 1 hour @ RT	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

cAMP Plate Layout

A1 Blank	A2 Std 1	A3 Std 5	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1 Blank	B2 Std 1	B3 Std 5	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1 TA	C2 Std 2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1 TA	D2 Std 2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1 NSB	E2 Std 3	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1 NSB	F2 Std 3	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1 Bo	G2 Std 4	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1 Bo	H2 Std 4	H3	H4	H5	H6	H7	H8	H	H10	H11	H12

Kit Lot No. ______ Exp. Date ______ Date ______ Tech. ______

1st Incub.: Start Time ______ Temp. ______ Notes:

End Time ______ Temp. ______

2nd Incub.: Start Time ______ Temp. ______

End Time ______ Temp. ______

For a full-sized version of this chart, please visit www.trevigen.com.

XVII. References

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XVIII. Related Products Available From Trevigen

2501-096-K	HT Direct Cyclic AMP Enzyme Immunoassay Kit	96 wells

Oxidative Damage Kits

4370-096-K	8-oxo-dG ELISA Kit	96 wells
7500-100-K	SOD Assay Kit	100 tests
7501-500-K	HT SOD Assay Kit	480 tests
7510-100-K	Glutathione Reductase Assay Kit	100 tests
7513-500-K	HT Glutathione Reductase Assay Kit	480 tests
7511-100-K	HT Glutathione Assay Kit	384 tests
7512-100-K	HT Glutathione Peroxidase Assay Kit	480 tests

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