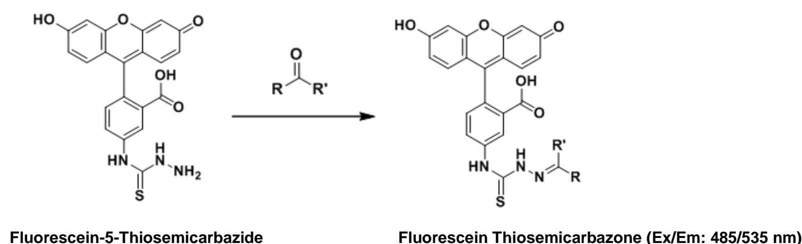
Protein Carbonyl Content Assay Kit (Fluorometric)

(Catalog # K563-100; 100 assays; Store at -20°C)

I. Introduction:

Protein carbonylation occurs when chronically elevated blood sugar levels create a microenvironment for sugars to covalently react with amino acids forming non-enzymatic adducts. Oxidative stress, the excessive production of ROS, can also give rise to the production of protein carbonyls. Over time, these two processess create Advanced Glycation End-products (AGEs) with proteins. The major functional outcome of protein-reactive AGEs is reduced enzymatic activity. Soluble AGEs can also bind and activate the receptor of advanced glycation end-products (RAGE). Receptor-dependent outcomes lead to tissue dysfunction, oxidative stress, and activation of monocytes, endothelial cells and mesangial cells. In Alzheimer's Disease (AD) protein carbonyls are a marker of oxidative stress in the brain and are elevated in serum and plasma proportional to the cognitive severity. BioVision's Protein Carbonyl Content Assay Kit uses Fluoroscein-5-Thiosemicarbazide (FTC), a fluorescent probe which covalently reacts with oxidized residues (i.e Cysteine, Lysine, Arginine, Histidine and Aspartic Acid) on proteins. The protein carbonyl content is determined by the generation of a stable fluorometric signal (Ex/Em 485/535 nm) and compared to the protein concentration determined in the BCA Assay to quantitate nmoles of carbonyl/mg protein. The kit is simple, requires no harsh chemicals, can quantitate carbonyls in serum or plasma and produces more reliable and reproducible results than the comparable colorimetric assays. It can detect carbonyl groups in samples with protein concentrations as low as 1 mg/ml!



Fluorescein-5-Thiosemicarbazide Fluorescein Thiosemicarbazone (Ex/Em: 485/535 nm)

II. Applications:

- Quantification of carbonyl content as a putative biomarker for research in disease areas such as diabetes, multiple sclerosis, and Alzheimer's disease.

III. Sample Types:

- Biological fluids such as serum, plasma, and cell lysate.

IV. Kit Contents:

Components	K563-100	Cap Code	Part Number
Protein Carbonyl Assay Buffer	20 ml	NM	K563-100-1
100% TCA Solution	20 ml	NM/Brown	K563-100-2
10% Streptozotocin Solution	1 ml	Blue	K563-100-3
6 M Guanidine Solution	20 ml	WM	K563-100-4
Sample Dilution Buffer	50 ml	NM	K563-100-5
FTC (10 mM in DMSO)	200 µl	Orange	K563-100-6
Positive Control Protein (10 mg/ml)	250 µl	Violet	K563-100-7

V. User Supplied Reagents and Equipment:

- Multi-well fluorescence microplate reader
- 96-well clear microtiter plates with flat bottom
- Isopropanol
- BCA Protein Assay Kit II (Cat # K813), or BCA Protein Assay Kit-Reducing Agent Compatible (Cat # K818-1000)
- dH_2O

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge vials prior to opening. Read entire protocol before performing the assay.

- **Protein Carbonyl Assay Buffer (AB), 10% Streptozotocin**, **Sample Dilution Buffer, FTC (10 mM in DMSO), Positive Control Protein (PCP) (10mg/ml):** Store at -20°C in dark.
- **100% TCA Solution**, **6 M Guanidine:** Store long term in -20°C. Upon receipt of kit, inspect bottles. If precipitation is observed, vortex the bottle or warm the content at 37°C until it completely dissolves. Before using TCA, dilute in dH_2O to 20% TCA and keep on ice. Bring other reagents to RT before use.

VII. Protein Carbonyl Content Assay Protocol:

1. **Sample Preparation:** The assay can be used on serum, plasma as well as other biological fluids when applicable. Use Protein Carbonyl Assay Buffer (AB) to adjust sample concentrations to 1-10 mg/ml prior to performing the assay. Dilute samples in AB and centrifuge to remove any insolubles. Use 50 µl of sample in a centrifuge tube. Prepare a reagent background control using 50 µl of AB. For Positive Control, dilute Positive Control Protein (PCP) to 5 mg/ml (50 µl of PCP with 50 µl of AB). Add 50 µl of 5 mg/ml PCP to an unused 1.5 ml centrifuge tube.

   Notes:

   a. Protein samples lower than 1 mg/ml should be concentrated with a 10 kD spin filter (Cat# 1997-25).

b. Nucleic acids can erroneously contribute to higher estimation of carbonyls. Samples containing significant nucleic acid content should be treated with Streptozotocin (10 µl per 100 µl sample). Incubate for 15 min at room temperature, centrifuge for 5 min x 10000 x *g* and transfer supernatant to a new tube. Measure 280/260 nm ratio to make sure it is greater than 1.

2. **Reaction Mix:** Dilute Fluorophore (FTC) 50 times with AB (*i.e.* for 5 samples add 5 µl of FTC to 245 µl of AB). Prepare sufficient reagent for the number of samples to be assayed (50 µl/assay). Add 50 µl of 0.2 mM FTC to each 50 µl sample in a 1.5 ml centrifuge tube. Allow fluorophore to react with sample overnight at RT, protect from light.
3. **Quantify Carbonyl Content:** On the next day, precipitate proteins by adding 200 µl of 20% ice-cold TCA/centrifuge tube. Incubate mixture 10 minutes on ice. Centrifuge for 10 min at 10000 x *g*, 4°C. Remove supernatant. Wash pellet with 200 µl of 100% ice-cold isopropanol (not provided). Manually break-up pellet with pipette tip. (*Small particles at this step improve solubility when 6 M Guanidine added*). Centrifuge samples (as above), remove supernatant. Repeat wash step for a total of three times and, allow pellet to air dry at RT (this may take 1 h). Resuspend pellet with 50 µl of 6 M Guanidine and incubate samples on a heat block at 50°C for 1-2 h to improve solubility of pellet. Cool down sample to Room Temperature. When pellet is completely dissolved, add 450 µl of Sample Dilution Buffer. Determine protein concentration in solubilized pellet (*i.e.* BCA Assay). Then, add 100 µl of each solubilized pellet sample/well in a 96-well clear microplate.
4. **Standard Curve Preparation:** Prepare a 25 µM solution of FTC by adding 2 µl of stock FTC (10 mM) to 798 µl of AB to create a working standard. Add 0, 2, 4, 6, 8, 10 µl of working standard solution to each well to create 0, 50, 100, 150, 200, 250 pmoles/well of FTC Standard. Adjust volume to 100 µl with AB. Standard Curve can be read in end point mode (i.e. at the final incubation time).
5. **Measurement:** Measure fluorescence of all standards, samples and background controls (Ex/Em 485/535 nm). *See Protocol Quick Guide below.*
6. **Calculations:** Subtract 0 FTC Standard reading from all readings. Plot the FTC Standard Curve. If sample background control is significant, then subtract reagent background control reading from sample reading. Apply corrected RFU to Standard Curve to get B pmol FTC in the sample well.

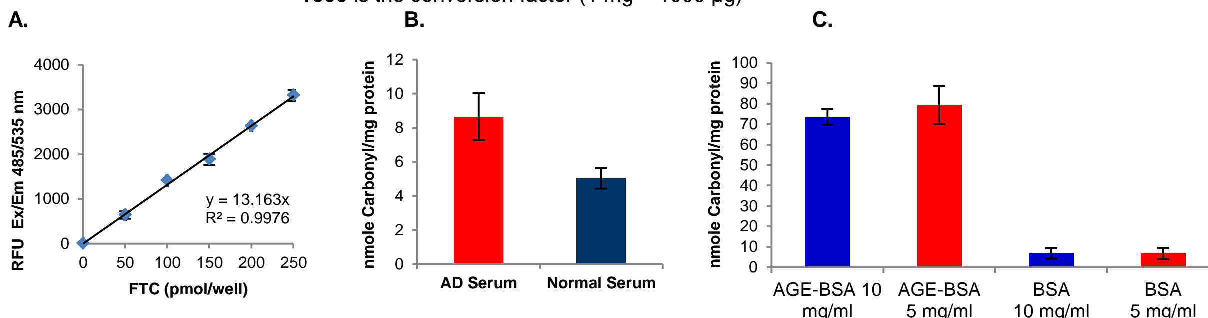
$$\textbf{Sample Protein Carbonyl Concentration (C)} = \frac{B*DF*sDF}{V}\left(\frac{pmol}{\mu l}\ \text{or}\ \mu M\right)$$

Where: **B** is amount of Protein Carbonyl in the sample well from Standard Curve (pmol)
V is the sample volume in the reaction well (100 µl)
DF is the Dilution Factor generated by the assay (*DF= 10*)
sDF is the Dilution Factor generated during sample preparation (*sDF= 1 if undiluted*)

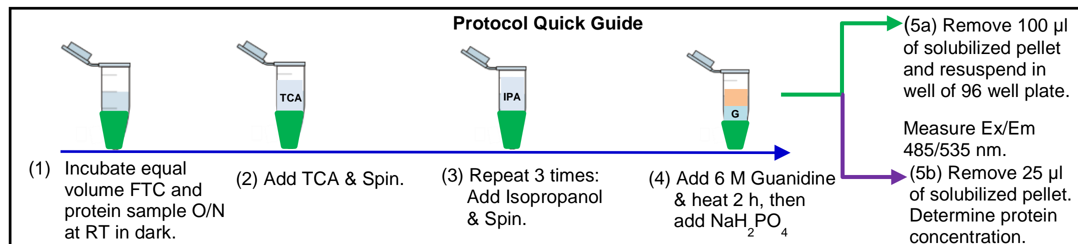
Alternatively, Protein Carbonyl content can be expressed in pmol Carbonyl/mg protein:

$$\textbf{Carbonyl Content} = \frac{C*1000}{p}\left(\frac{pmol}{mg\ protein}\right)$$

Where: **C** is Sample Protein Carbonyl Concentration (pmol/µl), see equation above
p is the Sample protein concentration (µg/µl, undiluted)
1000 is the conversion factor (1 mg ≡ 1000 µg)



Figures: **(A)** FTC Standard Curve. **(B)** Evaluation of carbonyl content in human serum: 50 µl of human serum from patients with Alzheimer's Disease (AD) or healthy individuals were assayed following kit protocols. **(C)** Comparison of AGE-BSA with untreated BSA illustrating the 7-fold increase of carbonyls on AGEs as compared to BSA.



VIII. Related Products

10 kDa spin filter (1997)
BCA Protein Assay Kit II (K813)
Protein Carbonyl Content Assay Kit (K830)

FOR RESEARCH USE ONLY! Not to be used on humans.