Isocitrate Colorimetric Assay Kit (#BN00880)

(Catalog #BN00880; 100 assays; Store Kit at +4° C)

I. Introduction:

Isocitric acid (HOOC-CHOH-CH (-COOH)-CH_2-COOH) is an intermediate of the Krebs TCA cycle, positioned between citrate and α-ketoglutarate. It is the branch point from which the glyoxylate shunt operates in plants and lower organisms. Isocitrate is found in substantial concentrations in many fruits and vegetables as well as in foods produced from these raw materials. In the TCA cycle, isocitrate is oxidized by isocitrate dehydrogenase (IDH) to α-ketoglutarate with the generation of NAD(P)H. Loss of NAD-IDH has been implicated as a potential causative factor in retinitis pigmentosa. Assay Genie's Isocitrate Assay Kit provides a simple, sensitive and rapid means of quantifying isocitrate in a variety of samples. In the assay, isocitrate is oxidized with the generation of NADPH which converts a nearly colorless probe to an intensely colored species with a λ_{max} of 450nm. The Isocitrate Assay Kit can detect 1 to 20 nmoles (~0.2 – 5 µg) of isocitrate.

II. Kit Contents:

Components	BN00880	Cap Code	Part Number
Isocitrate Assay Buffer	25 ml	WM	BN00880-1
Isocitrate Enzyme Mix	200 µl	Green	BN00880-2
Substrate Mix	lyophilized	Purple	BN00880-3
Isocitrate Standard (100mM)	100 µl	Yellow	BN00880-4

III. Storage and Handling:

Store kit at +4°C, protect from light. Warm Isocitrate Assay Buffer to room temperature before use. Briefly centrifuge all small vials prior to opening.

IV. Reagent Preparation and Storage Conditions:

Isocitrate Enzyme Mix: Ready to use as supplied. Use within two months.

Substrate Mix: Add 220 µl dH_2O and dissolve. Stable for 2 months at 4° C.

Isocitrate Standard: Ready to use as supplied. Keep cold while in use. Store at -20° C.

V. Assay Protocol:

1. Standard Curve Preparations:

Dilute Isocitrate Standard to 2 nmol/µl by adding 20 µl of the Standard to 980 µl of dH_2O, mix well. Add 0, 2, 4, 6, 8, 10 µl into a series of wells on a 96 well plate. Adjust volume to 50 µl/well with Assay Buffer to generate 0, 4, 8, 12, 16, 20 nmol/well of the Standard.

2. Sample Preparation:

Tissue 20 mg or cells (2 x 10^6) should be rapidly homogenized with 100 µl Isocitrate Assay Buffer. Centrifuge at 15,000 g for 10 min to remove cell debris. Enzymes in samples may interfere with the assay. We suggest deproteinizing your sample using a perchloric acid/KOH protocol or 10 kDa molecular weight cut off spin columns. Add 1-50 µl samples into duplicate wells of a 96-well plate and bring volume to 50 µl with Assay Buffer.

Food or Beverage samples: Most beverages can be used directly in the assay, with appropriate dilution. In general, samples should be spin filtered through a 10kDa MWCO filter. This will remove inhibitory substances, protein and most color. Solids should be processed by homogenizing 20 mg with 500µl distilled water, with mild heating for 30 min, then centrifuge 15,000x g, 10 min, take supernatant, spin filter and dilute appropriately for the assay. For all samples, we suggest testing several doses of your samples to ensure readings are within the standard curve range.

3. Develop:

Mix enough reagent for the number of samples and standards to be performed: For each well, prepare a total 50 µl Reaction Mix containing:

Isocitrate Assay Buffer	46 µl
Isocitrate Enzyme Mix	2 µl
Substrate Mix	2 µl

** NADH and NADPH can generate significant background in some instances. If interfering levels of these are suspected of being in the sample, a background control can be performed by running a parallel sample with the Isocitrate Enzyme Mix being omitted.

Add 50 µl of Reaction Mix to each well containing the Isocitrate Standard and test and background control samples.

4. Incubate for 30 min at 37° C, protect from light.

5. Measure OD at 450 nm with microplate reader

6. Calculation:

Correct background by subtracting the value of the 0 Isocitrate standard from all readings. (Note: The background reading can be significant and must be subtracted.) Plot the standard curve. Then apply the corrected sample readings to the standard curve to get Isocitrate amount in the sample wells.

The Isocitrate concentrations in the test samples:

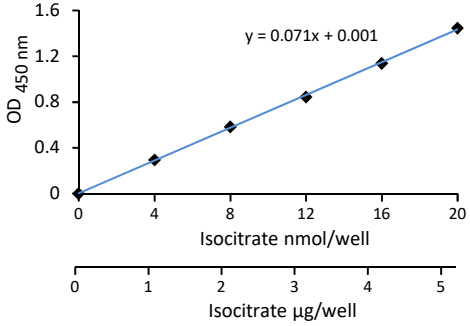
C = Ay/Sv (nmol/µl; or µmol/ml; or mM)

Where:

Ay is the amount of Isocitrate (nmol) in your sample from the standard curve.

Sv is the sample volume (µl) added to the sample well.

Isocitrate molecular weight: 192.12 g/mol



Isocitrate standard curve generated using this kit protocol.

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

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Samples were not deproteinized (if indicated in datasheet)	• Use the 10 kDa spin cut-off filter or PCA precipitation as indicated
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed, deproteinize samples
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		