SUCCINIC ACID

PRODUCT INSTRUCTIONS

SKU: 700004340
K-SUCC

01/24

For the assay of Succinic Acid (Succinate)

(20 Manual Assays per Kit) or
(270 Auto-Analyser Assays per Kit) or
(200 Microplate Assays per Kit)

Play Training Video

INTRODUCTION:

Succinic acid is found in all plant and animal materials as a result of the central metabolic role played by this dicarboxylic acid in the Citric Acid Cycle. Succinic acid concentrations are monitored in the manufacture of numerous foodstuffs and beverages, including wine, soy sauce, soy bean flour, fruit juice and dairy products (e.g. cheese). The ripening process of apples can be followed by monitoring the falling levels of succinic acid. The occurrence of > 5 mg/kg of this acid in egg and egg products is indicative of microbial contamination. Apart from use as a flavouring agent in the food and beverage industries, succinic acid finds many other non-food applications, such as in the production of dyes, drugs, perfumes, lacquers, photographic chemicals and coolants.

PRINCIPLE:

In the presence of COA, adenosine-5'-triphosphate (ATP), succinic acid (succinate) is converted to succinyl-CoA by the enzyme succinyl- CoA synthetase (SCS), with the concurrent formation of adenosine- 5'-diphosphate (ADP) and inorganic phosphate (Pi) (1).

(1) Succinate + ATP + CoA $\xrightarrow{\text{(SCS)}}$ succinyl-CoA + ADP + P_i

In the presence of pyruvate kinase, ADP reacts with phosphoenolpyruvate (PEP) to form pyruvate and ATP (2).

(2) ADP + PEP $\xrightarrow{\text{(PK)}}$ ATP + pyruvate

The pyruvate produced is reduced to lactate by lactate dehydrogenase (LDH) in the presence of reduced nicotinamide- adenine dinucleotide (NADH), with the production of NAD^+ (3).

(3) Pyruvate + NADH + H^+ $\xrightarrow{\text{(LDH)}}$ lactate + NAD^+

The amount of NAD+ formed in the above coupled reaction pathway is stoichiometric with the amount of succinic acid. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

Analysis of commercial succinic acid should yield recoveries of approx. 100% (w/w). Other than succinic acid, succinyl-CoA synthetase also reacts with itaconic acid. However, the level of itaconic acid in foodstuffs is so low that it does not interfere with the analytical results.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.128 mg/L of sample solution at the maximum sample volume of 2.00 mL (or to 2.56 mg/L with a sample volume of 0.1 mL). The detection limit is 0.256 mg/L, which is derived from an absorbance difference of 0.010 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 0.8 to 40 µg of succinic acid per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to a succinic acid concentration of approx. 0.128 to 0.256 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of succinic acid has been completed within the time specified in the assay (approx. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding succinic acid (approx. 20 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding succinic acid to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from both neogen.com and megazyme.com.

KITS:

Kits suitable for performing 20 assays in manual format (or 270 assays in auto-analyser format or 200 assays in microplate format) are available from Neogen. The kits contain the full assay method plus:

Bottle 1: Buffer (8 mL, pH 8.4) plus sodium azide (0.02% w/v) as a preservative. Store at 4°C. See individual label for expiry.

Bottle 2: (x2) NADH plus stabiliser. Store below -10°C. See individual label for expiry.

Bottle 3: (x2) ATP plus PEP and CoA. Store below -10°C. See individual label for expiry.

Bottle 4: Pyruvate kinase plus lactate dehydrogenase suspension, 0.55 mL. Store at 4°C. See individual label for expiry.

Bottle 5: Succinyl-CoA synthetase suspension (0.55 mL). Store at 4°C. See individual label for expiry.

Bottle 6: Succinic acid (~ 2 g). Store at 4°C. See individual label for expiry.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of **bottle 1** as supplied.

2. Dissolve the contents of one of **bottle 2** in 2.4 mL of distilled water. This is **NADH solution**. **Stable for ~ 4 weeks at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes). **Do not** dissolve the contents of the other bottle until required.

3. Dissolve the contents of one of **bottle 3** in 2.4 mL of distilled water. This is **ATP/PEP/CoA solution**. Divide into appropriately sized aliquots and store in polypropylene tubes below -10°C between use and keep cool during use if possible. **Do not** dissolve the contents of the other bottle until required. Once dissolved, the reagent is stable for > 4 weeks below -10°C.

4 & 5. Use contents of **bottles 4** and **5** as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position. **Swirl the bottle to mix contents before use.**

6. Accurately weigh approx. 200 mg of **bottle 6** to the nearest 0.1 mg into a 1 L volumetric flask. This is **standard solution**. Fill to the mark with distilled water and mix thoroughly. Store 10 mL aliquots of this solution below -10°C.

NOTE: The succinic acid standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of succinic acid is determined directly from the extinction coefficient of NADH (see page 6).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 100 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of Buffer 1 and of NADH and ATP/PEP/CoA solutions).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.74 mL
Sample solution: 0.8-40 µg of succinic acid per cuvette (in 0.10-2.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample	-	0.10 mL
bottle 1 (buffer)	0.20 mL	0.20 mL
NADH solution	0.20 mL	0.20 mL
ATP/PEP/CoA solution	0.20 mL	0.20 mL
bottle 4 (PK/L-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
bottle 5 (SCS)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min). If the reaction has not stopped after 6 min, continue to read the absorbances at 2 min intervals until the absorbances either remain the same, or decrease constantly over 2 min**.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

** if this “creep” rate is greater for the sample than for the blank, extrapolate the absorbances (sample and blank) back to the time of addition of suspension 5.

CALCULATION:

NOTE: These calculations can be simplified by using the ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{succinic acid}}$. The value of $\Delta A_{\text{succinic acid}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of succinic acid can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{\text{succinic acid}} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of succinic acid [g/mol]
Ɛ = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for succinic acid:

$$c = \frac{2.74 \times 118.09}{6300 \times 1.0 \times 0.1} \times \Delta A_{\text{succinic acid}} \quad [g/L]$$

$$= 0.5136 \times \Delta A_{\text{succinic acid}} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of succinic acid

$$= \frac{c_{\text{succinic acid}}\ [g/L \text{ sample solution}]}{\text{weight}_{\text{sample}}\ [g/L \text{ sample solution}]} \times 100 \quad [g/100\ g]$$

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for succinic acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of succinic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	**Volume**
distilled water **bottle 1** (buffer) **NADH solution** **ATP/PEP/CoA solution** **bottle 4** (PK/L-LDH)	20.55 mL 2.40 mL 2.40 mL (after adding 2.4 mL of H_2O to bottle 2) 2.40 mL (after adding 2.4 mL of H_2O to bottle 3) 0.24 mL
Total volume	27.99 mL

Preparation of R2:

Component	**Volume**
distilled water **bottle 5** (SCS)	3.55 mL 0.26 mL
Total volume	3.81 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 6 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	decrease
Linearity:	up to 343 mg/L of succinic acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for succinic acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of succinic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.274 mL
Linearity: 0.1-4 µg of succinic acid per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
bottle 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
NADH solution	0.020 mL	0.020 mL	0.020 mL
ATP/PEP/CoA solution	0.020 mL	0.020 mL	0.020 mL
bottle 4 (PK/L-LDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:			
bottle 5 (SCS)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min). If the reaction has not stopped after 6 min, continue to read the absorbances at 5 min intervals until the absorbances increase constantly over 5 min**.			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 5.

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L\ standard \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of succinic acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.8 and 40 µg. The sample solution must therefore be diluted sufficiently to yield a succinic acid concentration between 0.008 and 0.40 g/L.

Dilution Table

Estimated concentration of succinic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.40	No dilution required	1
0.40-4.0	1 + 9	10
4.0-40	1 + 99	100
> 40	1 + 999	1000

If the value of $\Delta A_{succinic\ acid}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

IMPORTANT NOTE: Users should perform in-house matrix validation work prior to routine use. This process will highlight any problematic matrices encountered. The below are suggested sample preparation examples only.

Samples containing high amounts of protein or fats may cause interference in target analyte determination and require sample clarification prior to analysis using Carrez Clarification reagents. These reagents are available to purchase separately from Neogen in the Carrez Clarification Kit (**K-CARREZ**). The **Carrez Clarification Kit** reagents should be diluted prior to use as described in the assay protocol and the procedure for clarification followed as described on page 3 of the assay protocol. The clarified sample solution prepared using the **Carrez Clarification Kit** can then be analysed for succinic acid content as described in this assay procedure.

2. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 8.4 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing significant amounts of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 8.4 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no SCS, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of Polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to room temperature and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively, use **K-CARREZ**.

SUGGESTED SAMPLE PREPARATION EXAMPLES:

(a) Determination of succinic acid in white wine.

No sample preparation is required. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(b) Determination of succinic acid in red wine.

Dispense 25 mL of sample into a 100 mL beaker and adjust the pH to 7-8 using 1 M NaOH. Adjust the volume to 50 mL with distilled water and add 0.5 g of PVPP. Stir for 5 min and filter an aliquot of the suspension through Whatman No. 1 (9 cm) filter paper. *Typically, a dilution of 1:2 and a sample volume of 0.1 mL are satisfactory.*

(c) Determination of succinic acid in whole liquid egg. Accurately weigh approx. 5 g of homogenised whole egg into a 50 mL volumetric flask, add 25 mL of distilled water and 1 drop of *n*-octanol. Mix and incubate for 15 min in a water bath (approx. 100°C). Cool to 20-25°C and clarify using Carrez clarification reagents (**K-CARREZ**) as described in the protocol. *Typically, no further dilution is required and a sample volume of 0.2-1.0 mL is satisfactory.*

(d) Determination of succinic acid in whole egg powder. Accurately weigh approx. 1 g of whole egg powder into a 50 mL volumetric flask, add 30 mL of distilled water and 1 drop of *n*-octanol. Mix and incubate for 15 min in a water bath (approx. 100°C). Cool to 20-25°C and clarify using Carrez clarification reagents (**K-CARREZ**) as described in the protocol. *Typically, no dilution is required and a sample volume of 0.2-1.0 mL is satisfactory.*

(e) Determination of succinic acid in cheese.
Accurately weigh approx. 5 g of cheese into a 100 mL volumetric flask, add approx. 80 mL of distilled water and incubate at 60°C for 15 min. Shake the flask several times. Cool to 20-25°C and adjust the volume to 100 mL. For the separation of fat, place the flask in a refrigerator or on ice for approx. 20 min and centrifuge an aliquot of the lower aqueous phase. *Typically, no dilution is required and a sample volume of 1.0 mL is satisfactory.*
NOTE: The final volume of the clarified supernatant will be approx. one quarter of the starting volume of the original sample. Therefore, adjust the volume of the starting material as required to obtain sufficient volume of clarified sample for the test

NOTE: If you have questions about these or other matrices, please contact your local sales representative for support.

REFERENCE:

Beutler, H. O. (1989). Succinate. "*Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VII**, pp. 25-33, VCH Publishers (UK) Ltd., Cambridge, UK.

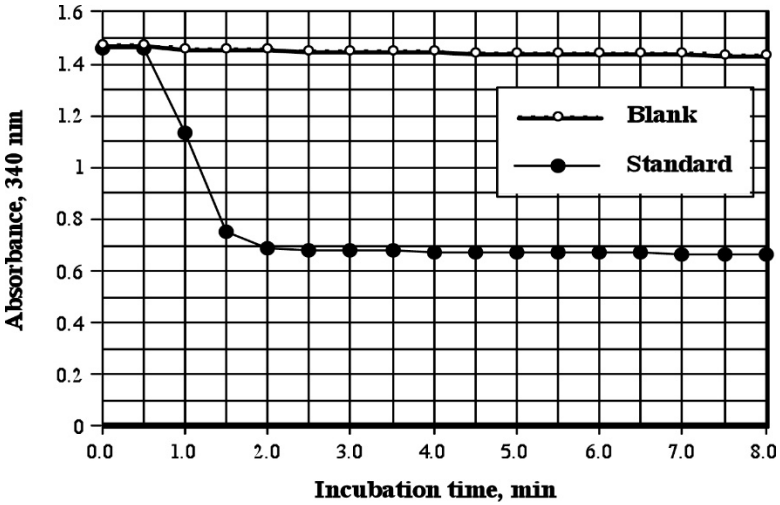

Figure 1. Decrease in absorbance at 340 nm on incubation of 40 µg of succinic acid with succinyl-CoA synthetase, pyruvate kinase and lactate dehydrogenase in the presence of NADH.

Contact us for more information: neogen.com/contact

Without guarantee

The information contained in this assay protocol is, to the best of our knowledge, true and accurate, but since the conditions of use are beyond our control, no warranty is given or is implied in respect of any recommendation or suggestions which may be made or that any use will not infringe any patents. It is the user's responsibility to perform in-house matrix validation work prior to routine use.