# Citrate Test Protocol

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## Information

### History

During an extensive study of the bacterial catabolism of organic acids, S. A. Koser identified citrate utilization as a defining characteristic that could be used to distinguish between coliforms such as*Enterobacter aerogenes* which occur naturally in the soil, on plants, and in aquatic environments and fecal coliforms such as *Escherichia coli* whose presence would be indicative of fecal contamination (5, 6). Koser's medium supported the growth of the *Enterobacter* group, organisms that could use citrate as the sole carbon and energy source. On the other hand, fecal coliforms such as *E. coli* were unable to grow in the medium.

One drawback to the original broth test was that a heavy inoculum could be misconstrued as a positive test for growth. As a solution to this conundrum, Simmons developed a new formulation that contained agar and the pH indicator bromothymol blue (BTB) (9). Simmons citrate medium is employed as an agar slant, and the solid surface makes it easier to determine whether or not a significant amount of growth has occurred. Because citrate utilization requires oxygen, the slant style will also increase the amount of growth. The increase in pH of the medium that occurs upon citrate breakdown can be detected by the concomitant change in the BTB color.

### Purpose

The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source (3, 7). A positive diagnostic test rests on the generation of alkaline by-products of citrate metabolism. The subsequent increase in the pH of the medium is demonstrated by the color change of a pH indicator.

The citrate test is often part of a battery of tests used to identify gram-negative pathogens and environmental isolates (10). For instance, test kits such as the API-20E (bioMerieux) and Enterotube II (BD Diagnostics) include citrate utilization medium as one of the diagnostic tests.

### Theory

The citrate test is commonly employed as part of a group of tests, the IMViC tests, that distinguish between members of the Enterobacteriaceae family based on their metabolic by-products (1, 2, 4). In the most common formulation, citrate is the sole source of carbon in the Simmons

citrate medium while inorganic ammonium salt ($NH_4H_2PO_4$) is the sole fixed nitrogen source (3, 4, 7, 8). When an organic acid such as citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates ultimately are produced (5, 7). The visible presence of growth on the medium and the change in pH indicator color due to the increased pH are the signs that an organism can import citrate and use it as a sole carbon and energy source; such organisms are considered to be citrate positive.

Citrate, a Krebs cycle (i.e., TCA cycle or citric acid cycle) intermediate, is generated by many bacteria; however, utilization of exogenous citrate requires the presence of citrate transport proteins (permeases) (7). Upon uptake by the cell, citrate is cleaved by citrate lyase to oxaloacetate and acetate. The oxaloacetate is then metabolized to pyruvate and $CO_2$.

citrate = oxaloacetate + acetate

oxalacetate = pyruvate + $CO_2$

Further metabolic breakdown is dependent upon the pH of the medium. Under alkaline conditions, pyruvate is metabolized to acetate and formate.

pyruvate = acetate + formate

At pH 7.0 and below, lactate and acetoin are also produced.

pyruvate = acetate + lactate + $CO_2$

pyruvate = acetoin + $CO_2$

The carbon dioxide that is released will subsequently react with water and the sodium ion in the medium to produce sodium carbonate, an alkaline compound that will raise the pH. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source. The bromothymol blue pH indicator is a deep forest green at neutral pH (Fig. 1). With an increase in medium pH to above 7.6, bromothymol blue changes to blue (Fig. 2).

Although uncommon, natural *E. coli* variants that are citrate positive have been isolated. Citrate-negative strains of *E. aerogenes* have also been found.

**RECIPES**

Simmons citrate medium (3, 4, 7, 8)

| Ingredient | Amount |
|---|---|
| Magnesium sulfate (heptahydrate) | 0.2 |

| | |
|---|---|
| Ammonium dihydrogen phosphate | 1.0 g |
| Dipotassium phosphate | 1.0 g |
| Sodium citrate (dehydrate) | 2.0 g |
| Sodium chloride | 5.0 g |
| Agar | 15.0 g |
| Bromothymol blue | 0.08 g |
| Deionized water | 1,000 ml |

Dissolve salts in deionized water. Adjust pH to 6.9. Add agar and BTB. Gently heat, with mixing, to boiling until agar is dissolved. Dispense 4.0 to 5.0 ml into 16-mm tubes. Autoclave at 121°C under 15 psi pressure for 15 minutes. Cool in slanted position (long slant, shallow butt). Tubes should be stored in a refrigerator to ensure a shelf life of 6 to 8 weeks. The uninoculated medium will be a deep forest green due to the pH of the sample and the bromothymol blue.

Simmons citrate medium is available both as a premixed powder and premade slants from a variety of suppliers.

**PROTOCOL** (3, 7, 8)

**A. Inoculation of medium**

Prepare medium as described above or as directed by the manufacturer. Allow tubes to warm up to room temperature prior to inoculation. Use a fresh (16- to 18-hour) pure culture as an inoculation source. Pick a single isolated colony and lightly streak the surface of the slant. A needle is the preferred sampling tool in order to limit the amount of cell material transferred to the agar slant. Avoid using liquid cultures as the inoculum source. Citrate utilization requires oxygen and thus screw caps, if used, should be placed loosely on the tube.

**B. Incubation conditions**

Incubate at 35°C (+/- 2°C) for 18 to 48 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.

**C. Interpretation of results**

Citrate positive: growth will be visible on the slant surface and the medium will be an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH

of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue (Fig. 2).

Citrate negative: trace or no growth will be visible. No color change will occur; the medium will remain the deep forest green color of the uninoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant (Fig. 1).


FIG. 1. Uninoculated Simmons citrate slant. The green color indicates that the medium pH is between 6.9 and 7.6. Except perhaps for a faint streak mark, a slant inoculated with a citrate-negative organism will appear identical to the uninoculated medium.


FIG. 2. Citrate-positive culture. Note the blue color of the medium and the faint white layer of growth on the slant surface.

**Alternate Methods to Test for Citrate Utilization**

Several citrate utilization media have been developed. One distinguishing factor amongst the methods is that some require that the

organism be able to use citrate in order to grow, while others have alternative carbon sources available. Koser's citrate medium (3), Simmons citrate medium, and Christensen's citrate medium are all commercially available.

Citrate is the only carbon source in both Koser's and Simmons media, thus growth in either medium indicates that the test organism has the ability to use citrate as its sole carbon source. Due to the absence of a pH indicator, the Koser formulation may be more prone to misinterpretation. The Simmons formulation is the one that is most commonly used in microbiology teaching labs as part of the IMViC protocol.

Christensen developed an alternative citrate test medium that does not require the organism to use citrate as a sole carbon source (7). Christensen's medium contains both peptone and cysteine. Thus citrate-negative bacteria can also grow on this medium. A positive reaction shows that the organism can use citrate but not necessarily as the sole carbon source (see below).

**Christensen's citrate sulfide medium (7)**

| Ingredient | Amount |
|---|---|
| Yeast extract | 0.5 g |
| Glucose | 0.2 g |
| Sodium citrate dihydrate | 3.0 g |
| Monopotassium phosphate | 1.0 g |
| Cysteine-HCl | 0.1 g |
| Iron (III) ammonium citrate (50/50) (optional) | 0.4 g |
| Sodium chloride | 5.0 g |
| Sodium thiosulfate pentahydrate (optional) | 0.08 g |
| Agar | 15.0 g |
| Phenol red | 0.012 g |

Dissolve components in 1,000 ml of deionized water. Adjust pH to 6.7. The rest of the medium preparation is the same as for Simmons citrate medium, as are the inoculation and incubation methodologies. In contrast to Simmons' method, Christensen's medium should support growth of both citrate utilizers and nonutilizers. A positive test would be indicated by a color change in the medium from yellow at pH 6.7 to red at more alkaline pH values. A citrate-negative culture would remain yellow. Christensen's medium can also be used to test for the generation of hydrogen sulfide ($H_2S$) by the organism. Any $H_2S$ produced would react with the iron in the medium to form a black precipitate.

## SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

## COMMENTS AND TIPS

1. It is essential that a light inoculum be used, because the presence of visible cell material may be considered a positive citrate test. Bacteria from a heavy inoculum may be misconstrued as a positive test.

2. Students may be instructed to use light and heavy inocula and compare the results, so that they can appreciate the importance of using a light inoculum for accurate interpretation of results.

3. As with any biochemical identification test, some natural isolates may give weak or nonstandard test results. Thus it is important to utilize multiple identification tests when attempting to characterize an unknown bacterium.

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