

## Dilution Method<sup>1</sup>

## Method 8043

**Scope and application:** For water and wastewater.

<sup>1</sup> Adapted from *Standard Methods for the Examination of Water and Wastewater* and from Klein, R.L.; Gibbs, C. *Journal of Water Pollution Control Federation*, 1979, 51(9), 2257.



### Test preparation

#### Before starting

This test is a 5-day test. Complete all the steps carefully to make sure that the test does not have to be done again.

Prepare the dilution water with a BOD Nutrient Buffer Pillow. Make sure that the dilution water for this test does not contain an oxygen demand or toxins. When incubated for 5 days at 20 °C, the dissolved oxygen concentration in the dilution water must not change by more than 0.2 mg/L.

Carbonaceous BOD (CBOD) can be determined by the addition of nitrification inhibitor. A test for CBOD is recommended for biologically-treated effluents, samples with bacterial seed, samples with biologically treated effluents and river water.

As an alternative, use the method [BOD calculation-Graphical Method](#) on page 6 to calculate the test results. The graphical calculation method is also a tool for troubleshooting problems in BOD measurements. The graphical calculation method is not approved for regulatory reporting.

Review the Safety Data Sheets (MSDS/SDS) for the chemicals that are used. Use the recommended personal protective equipment.

Dispose of reacted solutions according to local, state and federal regulations. Refer to the Safety Data Sheets for disposal information for unused reagents. Refer to the environmental, health and safety staff for your facility and/or local regulatory agencies for further disposal information.

#### Items to collect

Description	Quantity
BOD bottle, 300 mL, glass, with glass stoppers and plastic caps	6
BOD bottle cap, plastic	6
Dilution water (refer to <a href="#">Prepare the dilution water</a> on page 3)	varies
Pipet, seriological, 1 mL, 5 mL and 10 mL	1
Incubator	1
Nitrification Inhibitor (for the CBOD test only)	1 bottle

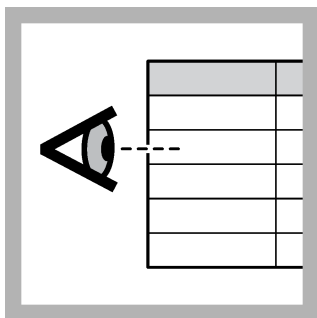
Refer to [Consumables and replacement items](#) on page 10 for order information.

#### Sample collection

The main consideration with sample collection is to prevent contamination of the sample with atmospheric oxygen.

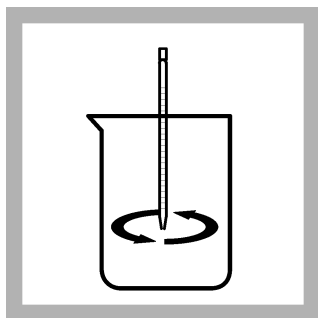
- Analyze the samples immediately. The samples cannot be preserved for later analysis.
- Collect samples in 300 mL glass BOD bottles. Completely fill the bottles.

## Test procedure

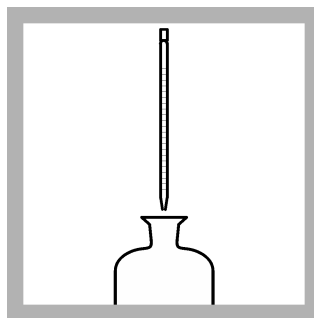


1. Identify five sample volumes to use for this test. Refer to [Select the sample volumes](#) on page 4.

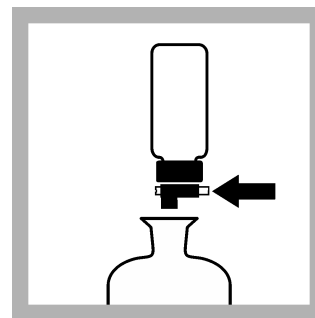
**Note:** If the minimum sample volume is 3 mL or more, determine the dissolved oxygen in the undiluted sample. Ignore this determination if sewage and settled effluents with a dissolved oxygen content near 0 mg/L are analyzed. If industrial effluents and disinfected samples are analyzed, refer to [Interferences](#) on page 8.



2. **Prepare the samples:** Gently stir the sample.



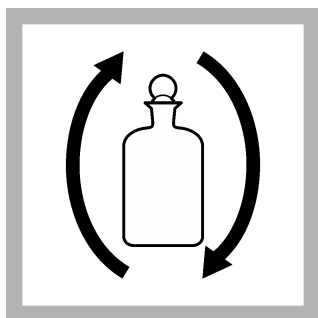
3. Use a pipet to add the sample volumes to five 300-mL BOD bottles.



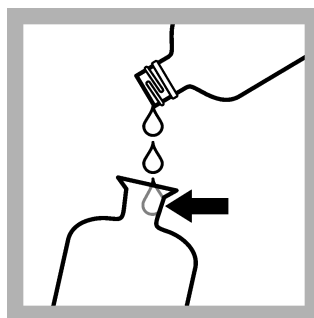
4. If the test is for CBOD, add two portions of Nitrification Inhibitor (approximately 0.16 g) to each bottle to prevent the oxidation of nitrogen compounds. Record the results as CBOD.



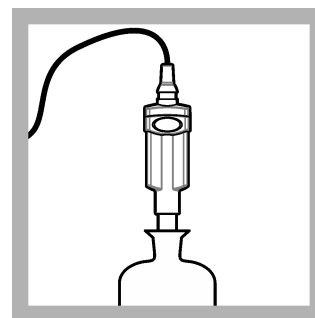
5. Fill each bottle with prepared dilution water. Refer to [Prepare the dilution water](#) on page 3. To prevent air bubbles, pour the water down the inner surface of the bottle.



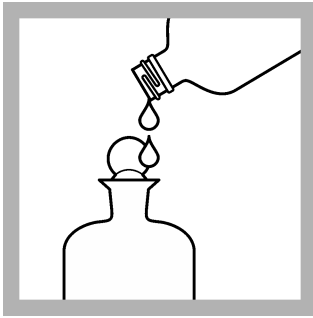
6. Carefully insert a stopper in each bottle to prevent trapped air bubbles. Push down on the stopper. Invert the bottles several times to mix.



7. **Prepare the blank:** Fill another 300-mL BOD bottle with the prepared dilution water. To prevent air bubbles, pour the water down the inner surface of the bottle.



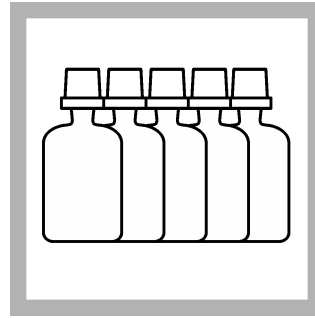
8. Use a probe or use a titration procedure to measure the dissolved oxygen concentration in each bottle. If titration is used for the measurement, prepare two sets of BOD bottles. Make sure to measure the dissolved oxygen of the blank.



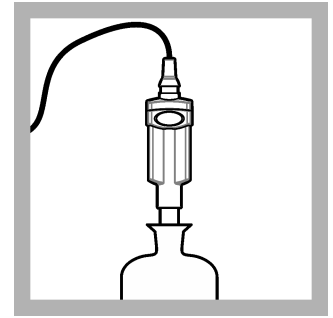
**9.** Carefully insert a stopper in each of the prepared sample bottles to prevent trapped air bubbles. Add dilution water above the stopper of the BOD bottles to make a water seal.



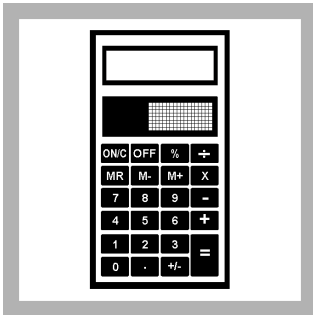
**10.** Add a cap to each bottle to prevent evaporation.



**11.** Keep the prepared sample bottles in an incubator at 20 °C (68°F). Do not move the prepared sample bottles for 5 days.



**12.** After 5 days, measure the remaining dissolved oxygen in each of the prepared samples. For accurate results, the prepared samples must contain a minimum 1.0 mg/L DO concentration after incubation.



**13.** Calculate the BOD value. Refer to [BOD calculation—Standard Methods](#) on page 6 or [BOD calculation-Graphical Method](#) on page 6.

## Prepare the dilution water

Make sure that no source of oxygen demand or toxins are added when the dilution water is prepared.

Items to collect:

- Dilution water (refer to the dilution water guidelines)
- BOD Nutrient Buffer Pillow<sup>1</sup>
- Raw sewage<sup>2</sup> for the bacterial seed, 3 mL (if the sample is low in bacteria)

<sup>1</sup> Different sizes are available for different quantities of water (e.g., 3 L and 6 L).

<sup>2</sup> Keep the raw sewage at 20 °C (68°F) and do not move for 24–36 hours before use. Pipet from the upper portion of the sewage.

---

## Dilution water guidelines

- For the best results, use distilled water from an alkaline permanganate distillation.
- Use high-quality water that does not contain organic compounds or toxic compounds (e.g., chlorine, copper and mercury).
- Do not use deionized water from ion exchange columns. The resins in the cartridges (mostly new cartridges) occasionally release organic materials that have an oxygen demand. In addition, bacteria can grow on the columns, which adds contamination to the dilution water.
- The dissolved oxygen concentration of the dilution water must not change by more than 0.2 mg/L when incubated for 5 days at 20 °C (68 °F).

Prepare the dilution water as follows:

1. Keep the dilution water in clean jugs in an incubator at 20 °C (68°F). Shake the jugs to saturate the water with air. As an alternative, loosely put the cap on the jugs and wait a minimum of 24 hours before use.
2. (Optional) Use a small aquarium pump or an air compressor to saturate the water with air. Make sure to use filtered air. Make sure that the filter does not grow bacteria.
3. Shake the BOD Nutrient Buffer Pillow to mix the contents.
4. Add the contents of the BOD Nutrient Buffer Pillow to the distilled water.
5. Put the cap on the jug. Shake the jug vigorously for 1 minute to dissolve the nutrients and to saturate the water with air.
6. If the sample is known to be low in bacteria (e.g., industrial waste or sewage that has been disinfected), immediately before the test, add 3 mL of raw sewage to each liter of the dilution water.

Measure the BOD of the raw sewage collected. The BOD of the raw sewage will be subtracted from the BOD of the sample.

## Conventional method (optional)

As an alternative, prepare the dilution water with the conventional method as follows:

1. Pipet 1 mL of each of the solutions that follow per liter of distilled water at 20 °C:  
**Note:** *Be careful to prevent contamination of the solutions.*
  - Calcium Chloride Solution
  - Ferric Chloride Solution
  - Magnesium Sulfate Solution
  - Phosphate Buffer Solution<sup>3</sup>
2. Put the cap on the bottle. Shake the bottle vigorously for 1 minute.

## Select the sample volumes

Select the five sample volumes to use for this test. The sample volumes change based on the sample BOD and the altitude of the laboratory.

- High BOD samples (e.g., raw sewage)—Use small sample volumes so that all the oxygen is not consumed.
- Low BOD samples—Use large sample volumes so that sufficient oxygen is consumed to give an accurate result.

At higher altitudes, the amount of oxygen that dissolves in water decreases, so less oxygen is available to microorganisms. Refer to [Table 1](#).

**Note:** *At least 2.0 mg/L of dissolved oxygen (DO) must be consumed during the test and at least 1.0 mg/L DO must be in the BOD bottle after the test.*

1. Identify the minimum sample volume. Refer to [Table 2](#).

---

<sup>3</sup> Keep the phosphate buffer solution in a refrigerator to decrease the rate of biological growth.

For example, if a sewage sample contains approximately 300 mg/L BOD, the minimum sample volume is 2 mL. If the sewage effluent contains approximately 40 mg/L BOD, the minimum sample volume is 15 mL.

- Identify the maximum sample volume. Refer to [Table 3](#).

For example, if the sample contains approximately 300 mg/L BOD and the laboratory altitude is 305 m (1000 ft), the maximum sample volume is 8 mL. If the sample contains approximately 40 mg/L BOD and the laboratory altitude is 305 m (1000 ft), the maximum sample volume is 60 mL.

- Select three other sample volumes between the minimum and maximum volumes so that there are five sample volumes total.

**Table 1 Oxygen values at different altitudes—20 °C (68 °F)**

Altitude	Oxygen value in water saturated with air	Altitude	Oxygen value in water saturated with air
Sea level	9.2 mg/L	1219 m (4000 ft)	7.9 mg/L
305 m (1000 ft)	8.9 mg/L	1524 m (5000 ft)	7.6 mg/L
610 m (2000 ft)	8.6 mg/L	1829 m (6000 ft)	7.4 mg/L
914 m (3000 ft)	8.2 mg/L		

**Table 2 Minimum sample volume**

Sample type	BOD (mg/L)	Volume (mL)	Sample type	BOD (mg/L)	Volume (mL)
Strong trade waste	600	1	Oxidized effluents	50	12
Raw and settled sewage	300	2		40	15
	200	3		30	20
	150	4		20	30
	120	5		10	60
	100	6	Polluted river waters	6	100
	75	8		4	200
60	10	2		300	

**Table 3 Maximum sample volume**

mg/L BOD—Sea level	mg/L BOD—305 m (1000 ft)	BOD—1524 m (5000 ft)	Volume (mL)
2460	2380	2032	1
1230	1189	1016	2
820	793	677	3
615	595	508	4
492	476	406	5
410	397	339	6
304	294	251	8
246	238	203	10
205	198	169	12
164	158	135	15
123	119	101	20
82	79	68	30
41	40	34	60
25	24	21	100

**Table 3 Maximum sample volume (continued)**

mg/L BOD—Sea level	mg/L BOD—305 m (1000 ft)	BOD—1524 m (5000 ft)	Volume (mL)
12	12	10	200
8	8	7	300

**BOD calculation—Standard Methods**

Use the Standard Methods calculation when the results will be given to a regulatory agency. Give the results as CBOD<sub>5</sub> if nitrification inhibitor was added in the test.

When a bacterial seed is not added to the dilution water, calculate the BOD as follows:

$$\text{BOD}_5 \text{ mg/L} = (D_1 - D_2) \div P$$

When a bacterial seed is added to the dilution water, calculate the BOD as follows:

$$\text{BOD}_5 \text{ mg/L} = ((D_1 - D_2) - (B_1 - B_2) \times f) \div P$$

Where:

BOD<sub>5</sub> = BOD value from the 5-day test (mg/L)

D<sub>1</sub> = DO of the prepared sample immediately after preparation (mg/L)

D<sub>2</sub> = DO of the prepared sample after incubation in mg/L

P = Decimal volumetric fraction of sample used

B<sub>1</sub> = DO of the bacterial seed control immediately after preparation (mg/L)

B<sub>2</sub> = DO of bacterial seed control after 5-days at 20 °C (68 °F) in mg/L

f = ratio of the bacterial seed in the diluted sample to the bacterial seed in the bacterial seed control. f = (% seed in diluted sample) ÷ (% seed in seed control)

OR

If the bacterial seed material is added directly to sample or to the bacterial seed control bottles:

$$f = (\text{volume of the bacterial seed in the diluted sample}) \div (\text{volume of the bacterial seed in the bacterial seed control})$$

Averaged results are satisfactory if all the criteria that follows is true for more than one of the sample dilutions:

- The remaining DO is at least 1 mg/L.
- The final DO value is at least 2 mg/L less than the initial DO value.
- Toxicity at higher sample concentrations is not seen.
- There are no obvious anomalies.

**BOD calculation-Graphical Method**

<b>NOTICE</b>
Do not use the Graphical Method when the results must be given to a regulatory agency.

1. On a graph, record on the y-axis the remaining dissolved oxygen (DO) (mg/L) in each of the prepared samples after 5 days. Record the sample volume (mL) on the x-axis. Draw the best straight line through the plotted points. Refer to [Figure 1](#).

**Note:** At least three points should be on the graph line or very near to the graph line. Ignore an error point if seen at this time. For unseeded dilution water, the graph line should cross the mg/L oxygen remaining scale near or at less than the oxygen saturation value for the altitude of the laboratory as shown in [Prepare the dilution water on page 3](#).

2. To calculate the BOD, use the equation that follows, which is mathematically the same as the BOD equation. Refer to [BOD calculation—Standard Methods](#) on page 6.

$$\text{mg/L BOD} = (A \times 300) - B + C$$

Where:

A = the slope of the graph line. The slope of the graph line is equal to the mg/L DO consumed for each mL of sample. Select a point on the line and subtract the mg/L

---

DO remaining at that point from the mg/L DO where the line crosses with the DO scale (Y-intercept, mg/L DO remaining). Divide the difference by the mL of sample at the point selected.

300 = the volume of the BOD bottle (300 mL)

B = the Y-intercept. The Y-intercept is the DO value where the line crosses the “DO remaining” scale. (The Y-intercept should be very near to the actual dilution water blank value.)

C = the sample DO. The sample DO is the DO of the undiluted sample.

A different way to write this equation is:

$$\text{mg/L BOD} = (\text{slope} \times 300) - \text{Y-intercept} + \text{sample DO}$$

**Note:** If the best straight line is supplied by linear regression through the use of a calculator, change the sign (-) of the slope (+ slope) before the slope is multiplied by 300.

For example:

The mg/L DO remaining was determined for a series of four dilutions of domestic sewage after 5 days of incubation. The results are given in [Table 4](#).

If a set of BOD dilutions is done correctly with a homogeneous sample, a graph of the mg/L DO remaining versus the sample volume results in a straight line. The Y-intercept value is equal to the DO content of the dilution water after the 5-day incubation. But the Y-intercept value is not actually measured.

In this example, the Y-intercept is equal to 9.0 mg/L and the DO of the domestic sewage sample is thought to be zero. Refer to [Table 4](#). If another type of sample is used, measure the DO of an undiluted sample by the Winkler titration or potentiometrically. The slope in the example is calculated as follows:

$$(\text{DO value at Y-intercept} - \text{DO value at Point A}) \div (\text{sample volume at Point A} - \text{sample volume at Y-intercept})$$

$$(9.0 \text{ mg/L} - 3.0 \text{ mg/L}) \div 8 \text{ mL} - 0 \text{ mL} = 0.75 \text{ mg/L per mL} = \text{slope}$$

**American Public Health Association formula**—The calculation for BOD can be written as follows (not approved for reporting purposes):

$$\text{Slope} = (\text{mg/L DO remaining with a smaller sample volume} - \text{mg/L DO remaining with a larger sample volume}) \div$$

$$(\text{mL of a larger sample volume} - \text{mL of a smaller sample volume})$$

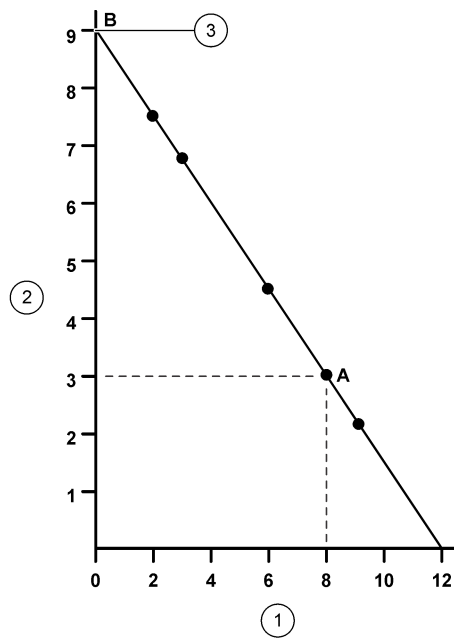
$$\text{slope} \times 300 - \text{DO}_D + S = \text{mg/L BOD}$$

Where:

$\text{DO}_D$  = mg/L DO of dilution water

S = mg/L DO of sample

**Figure 1 DO per mL of sample**



1 mL of sample	2 mg/L DO remaining	3 Y-intercept
----------------	---------------------	---------------

**Table 4 Sample volume versus DO remaining**

Sample volume	DO remaining
2.0 mL	7.50 mg/L
3.0 mL	6.75 mg/L
6.0 mL	4.50 mg/L
9.0 mL	2.25 mg/L

## Interferences

To get good BOD results, special handling is necessary to analyze chlorinated and industrial effluents. Usually, careful experimentation with the sample shows the changes that should be made to the test procedure. Toxins in the sample have an adverse effect on any microorganisms in the sample, which results in lower BODs.

The substances in [Table 5](#) interfere in the determination of oxygen demand at the given concentrations.

**Table 5 Interfering substances**

Interfering substance	Interference level
Chlorine	Small quantities of residual chlorine—Let the sample sit for 1 to 2 hours at room temperature. Larger quantities of chlorine—Refer to <a href="#">Remove chlorine from the sample</a> on page 9.
Phenols	Dilute the sample with high quality distilled water. As an alternative, acclimatize the bacterial seed used in the dilution water to tolerate such materials. Refer to <a href="#">Acclimatize the bacterial seed</a> on page 9.
Heavy metals	
Cyanide	
Highly buffered samples or extreme sample pH	Less than pH 6.5 or more than pH 7.5 interfere. Adjust to pH 7.2 with acid (Sulfuric Acid, 1 N or Phosphate Buffer Solution) or base (Sodium Hydroxide, 1 N).
Cold temperature	Cold samples can be supersaturated with oxygen and will have low BOD results. Fill a 1-liter (1-quart) bottle ½ full with cold sample. Shake the bottle vigorously for 2 minutes. Let the sample temperature increase to 20 °C (68 °F).



---

## Remove chlorine from the sample

Items to collect:

- 250-mL Erlenmeyer flask
  - 10-mL serological pipet and a pipet filler
  - 25-mL buret
  - 0.020 N Sulfuric Acid Standard Solution, 10 mL
  - 100-g/L Potassium Iodide Solution, 10 mL
  - 0.025 N Sodium Thiosulfate Standard Solution, 25 mL
  - Starch Indicator Solution, 3 full droppers
1. Add 100 mL of sample to a 250-mL Erlenmeyer flask.
  2. Use a 10-mL serological pipet and a pipet filler to add 10 mL of 0.020 N Sulfuric Acid Standard Solution to the flask.
  3. Use a 10-mL serological pipet and a pipet filler to add 10 mL of 100-g/L Potassium Iodide Solution to the flask.
  4. Add 3 full droppers of Starch Indicator Solution. Swirl to mix.
  5. Fill a 25-mL buret with 0.025 N Sodium Thiosulfate Standard Solution.
  6. Titrate the sample from dark blue to colorless.
  7. Calculate the amount of 0.025 N Sodium Thiosulfate Standard Solution to add to the sample.  
$$\text{mL 0.025 N Sodium Thiosulfate Standard Solution} = (\text{mL titrant used} \times \text{volume of remaining sample}) \div 100$$
  8. Add the calculated quantity of 0.025 N Sodium Thiosulfate Standard Solution to the sample.
  9. Mix fully. Wait 10–20 minutes before the test is done.

## Acclimatize the bacterial seed

1. Fill a 4-liter (1-gallon) stainless steel or plastic container with domestic sewage.
2. Aerate the sewage for 24 hours.
3. Let the heavier material collect on the bottom for 1 hour.
4. Use a siphon to remove 3 liters (3 quarts) of the material from the top and discard.
5. Fill the container with a mixture of 90% sewage and 10% wastes that contain the toxic material.
6. Aerate for 24 hours.
7. Do steps 3–5 again with more and more quantities of waste until the container holds 100% toxic waste material.

## Accuracy check

### Standard solution method

Use the standard solution method to validate the test procedure and the instrument.

Items to collect:

- 300-mg/L BOD Standard Solution<sup>4</sup>, Voluette Ampule, 10 mL
- Seeded dilution water
- Four 300-mL BOD bottles
- Pipet, volumetric, Class A, 1.0–4.0 mL
- TenSette Pipet

---

<sup>4</sup> 300-mg/L of glucose and 300-mg/L of glutamic acid

1. Use the TenSette pipet to add 1.00, 2.00, 3.00 and 4.00 mL of the standard solution to four 300-mL BOD bottles.
2. Fill each bottle with the seeded dilution water. Refer to [Prepare the dilution water](#) on page 3. To prevent air bubbles, let the water flow over and down the outer surface of the bottle.
3. Use the test procedure to measure the DO concentration of the diluted standard solutions and then again after 5 days. Do not add Nitrification Inhibitor. Record the values.
4. Calculate the BOD value.
5. Divide the calculated BOD value by 2. The expected result when compared to Standard Methods is 198 ( $\pm 30.5$ ) mg/L.

**Note:** The ampule includes 2 mL of 450 mg/L Glucose plus Glutamic Acid (GGA). Pour all the ampule is equivalent to add 6 mL of 150 mg/L solution with the Standard Methods. Calculate the BOD concentration as though there were 6 mL added to the bottle instead of 2 mL. The dilution factor for this standard is 50x.

## Summary of method

Biochemical oxygen demand (BOD) is a measurement of the oxygen requirements of municipal and industrial wastewaters and sewage. The test results are used to calculate the effect of waste discharges on the oxygen resources of the receiving waters. The BOD test gives a limited value in the measurement of the actual oxygen demand because the environmental factors (e.g., temperature change, biological population, water movement, sunlight, oxygen concentration and other factors) cannot be simulated accurately in the laboratory. The BOD test is a very important value after patterns of oxygen uptake for a specified effluent and receiving water are identified.

For this test, a sealed wastewater sample (or a prepared dilution) is incubated for the standard 5-day period. Then, the change in dissolved oxygen content is identified. The BOD value is calculated from the results of the dissolved oxygen tests.

## Consumables and replacement items

### Required reagents

Description	Quantity/Test	Unit	Item no.
BOD Nutrient Buffer Pillow, 3 mL (for 3 L of dilution water)	1 pillow	50/pkg	1486166

### Required apparatus

Description	Quantity/test	Unit	Item no.
BOD bottle with glass stopper, 300 mL	6	each	62100
BOD bottle cap, plastic	6	6/pkg	241906
Pipet, serological, 1 mL, glass	1	50/pkg	2093135
Pipet, serological, 5 mL	1	each	53237
Pipet, serological, graduated, 10 mL	1	each	53238
Pipet filler, safety bulb	1	each	1465100

### Recommended standards

Description	Unit	Item no.
BOD Standard Solution, Voluette® Ampule, 300 mg/L, 10 mL	16/pkg	1486510
ez GGA BOD Standard Solution, 300 mg/L, 2-mL ampules	20/pkg	2514420

## Optional reagents and apparatus

Description	Unit	Item no.
BOD bottle with glass stopper, 300 mL	6/pkg	62106
BOD bottle with glass stopper, 300 mL	24 pkg	62124
BOD Nutrient Buffer Pillow, 0.5 mL (for 300 mL of dilution water)	50/pkg	1416066
BOD Nutrient Buffer Pillow, 3 mL (for 3 L of dilution water)	50/pkg	1486166
BOD Nutrient Buffer Pillow, 4 mL (for 4 L of dilution water)	50/pkg	2436466
BOD Nutrient Buffer Pillow, 6 mL (for 6 L of dilution water)	50/pkg	1486266
BOD Nutrient Buffer Pillow, 19 mL (for 19 L of dilution water)	25/pkg	1486398
Calcium Chloride Solution, APHA, for BOD	500 mL	42849
Clippers	each	96800
Ferric Chloride Solution, APHA, for BOD	1 L	42953
Flask, Erlenmeyer, 250 mL	each	50546
Magnesium Sulfate Solution, APHA, for BOD	500 mL	43049
Nitrification Inhibitor	35 g	253335
Nitrification Inhibitor	500 g	253334
Nitrification Inhibitor, dispenser cap	each	45901
Phosphate Buffer Solution, APHA, for BOD, pH 7.2	500 mL	43149
Potassium Iodide Solution, 100 g/L	500 mL	1228949
Probe clips, color-coded, for IntelliCAL probes	50/pkg	5818400
Sodium Hydroxide, pellets, ACS	500 g	18734
Sodium Hydroxide Standard Solution, 1.00 N	100 mL MDB	104532
Sodium Thiosulfate Standard Solution, 0.025 N	1 L	35253
Sodium Thiosulfate Standard Solution, 0.1 N	100 mL	32332
Starch Indicator Solution	100 mL MDB	34932
Sulfuric Acid Standard Solution, 0.020 N	1 L	20353
Sulfuric Acid Solution, 1.00 N	1000 mL	127053



**FOR TECHNICAL ASSISTANCE, PRICE INFORMATION AND ORDERING:**

In the U.S.A. – Call toll-free 800-227-4224

Outside the U.S.A. – Contact the HACH office or distributor serving you.

On the Worldwide Web – [www.hach.com](http://www.hach.com); E-mail – [techhelp@hach.com](mailto:techhelp@hach.com)

**HACH COMPANY**  
WORLD HEADQUARTERS  
Telephone: (970) 669-3050  
FAX: (970) 669-2932