



## CONTENTS

<b>I. What a Buffer System Is and How It Works</b>	<b>1</b>
<b>II. What Makes a "Good" Buffer</b>	<b>1</b>
<b>III. Preparing Buffers</b>	<b>2</b>
A. Prepare Buffers at the Appropriate Temperature and Concentration.	2
B. Adjust the pH of the Buffer System Correctly.	2
C. Take Care of and Use the pH Meter Correctly.	2
D. Random Tips about Buffer Preparation	2
<b>IV. Appendix A: The Henderson-Hasselbalch Equation</b>	<b>3</b>
<b>V. Appendix B: Composition and Preparation of Common Buffers and Solutions</b>	<b>4</b>
A. Preparation of Bicarbonate-Carbonate Buffer (pH 9.2-10.8)	4
B. Preparation of Citrate Buffer (pH 3.0-6.2)	4
C. Preparation of Phosphate Buffer (pH 5.8-8.0 at 25°C)	4
D. Composition of Additional Buffers and Solutions	4
<b>VI. References</b>	<b>5</b>

## I. What a Buffer System Is and How It Works

Buffers often are overlooked and taken for granted by laboratory scientists until the day comes when a bizarre artifact is observed and its origin is traced to a bad buffer. Although mistakes in the composition of buffers have led occasionally to discoveries such as the correct number of human chromosomes (Arduengo, 2010), using the proper buffer, correctly prepared, can be key to success in the laboratory.

Most simply, a buffer functions to resist changes in hydrogen ion concentration as a result of internal and environmental factors. However, biologists often think of buffers as doing much more: providing essential cofactors for enzymatically driven reactions, critical salts, and even essential nutrients for cells and tissues. However, when the basic function of a buffer system, resisting changes in hydrogen ion concentration, is overlooked, experimental artifacts and other problems soon follow. Here we examine the basic chemistry of buffer systems and how that chemistry applies to reactions in experimental biological systems.

Buffers consist of a weak acid (HA) and its conjugate base (A<sup>-</sup>) or a weak base and its conjugate acid. Weak acids and bases do not completely dissociate in water, and instead exist in solution as an equilibrium of dissociated and undissociated species. Consider acetic acid. In solution acetate ions, hydrogen ions and undissociated acetic acid exist in equilibrium. This system is capable of absorbing either H<sup>+</sup> or OH<sup>-</sup> due to the reversible nature of the dissociation of acetic acid (HAc). HAc can release H<sup>+</sup> to neutralize OH<sup>-</sup> and form water. The conjugate base, A<sup>-</sup>, can react with H<sup>+</sup> ions added to the system to produce acetic acid. In this way, pH is maintained as the three species constantly adjust to restore equilibrium.

All buffers have an optimal pH range over which they are able to moderate changes in hydrogen ion concentration. This range is a factor of the dissociation constant of the acid of the buffer (K<sub>a</sub>) and is generally defined as the pK<sub>a</sub> (-logK<sub>a</sub>) value plus or minus one pH unit. pK<sub>a</sub> can be determined using the Henderson-Hasselbalch equation (Appendix A).

## II. What Makes a "Good" Buffer

In 1966, Norman Good and colleagues set out to define the best buffers for biochemical systems (Good *et al.* 1966). Good set forth several criteria for such buffers:

- **A pK<sub>a</sub> between 6 and 8.** Most biochemical experiments have an optimal pH in the range of 6–8. The optimal buffering range for a buffer is the dissociation constant of the weak acid component of the buffer (pK<sub>a</sub>) plus or minus pH unit.
- **Solubility in water.** Biological reactions, for the most part, occur in aqueous environments, and the buffer should be water-soluble for this reason.

- **Exclusion by biological membranes.** This is not important for all biochemical reactions. However, if this is an important criterion for your particular experiment, it is helpful to remember that zwitterionic buffers (positive and negative charges on different atoms within the molecule) do not pass through biological membranes. Examples of zwitterionic buffers include MOPS and HEPES; Tris and phosphate buffers do not isomerize into zwitterions.
- **Minimal salt effects.** In other words, the buffer components should not interact or affect ions involved in the biochemical reactions being explored.
- **Minimal effects on the dissociation from changes in temperature and concentration.** Usually there is some change in the dissociation with a change in concentration. If this change is small, stock solutions usually can be diluted without changing the pH. However, with some buffers, changes in concentration have more effect on dissociation, and stock solutions cannot be diluted without significantly affecting pH. For instance, the pH of Tris decreases approximately 0.1 pH unit per tenfold dilution, and the pH could change dramatically if you dilute a working solution and are at the limits of the optimal buffering range of the Tris (optimal buffering range pH 7.3–9.3 at 20°C). *Note that Tris is not one of Good's buffers.* Temperature changes can be a problem too. Tris exhibits a large shift in dissociation with a change in temperature. For example, if you prepare a Tris buffer at pH 7.0 at 4.0°C and perform a reaction in that same buffer at 37°C, the pH will drop to 5.95. If you have a Tris buffer prepared at 20°C with a pK<sub>a</sub> of 8.3, it would be an effective buffer for many biochemical reactions (pH 7.3–9.3), but the same Tris buffer used at 4°C becomes a poor buffer at pH 7.3 because its pK<sub>a</sub> shifts to 8.8. So the take-home message: **Make the buffer at the temperature you plan to use it**, and if your experiment involves a temperature shift, select a buffer with a range that can accommodate any shift in dissociation as a result.
- **Minimal interactions between buffer components and critical reaction components.** If a complex forms between the buffer and a required cofactor, say a metal cation like zinc or magnesium, your reaction also might be compromised. For example calcium precipitates as calcium phosphate in phosphate buffers. Not only would any Ca<sup>2+</sup>-requiring reactions be compromised, but the buffering capacity of the phosphate buffer also is affected. Having excessive amounts of a chelating agent in the buffer for an enzymatically driven reaction could cause problems (e.g., a high concentration of EDTA in a PCR amplification). Citrate is a calcium chelator, so avoid citrate buffers in situations where calcium concentrations are critical. Tris buffers again give us problems because Tris contains a reactive amine group. If you are trying to

make Tris buffer that is RNase-free, the amine group on the Tris molecule will react with diethylpyrocarbonate (DEPC), the chemical typically used to pretreat aqueous solutions used for RNA work. So, do not DEPC-treat Tris-containing solutions. HEPES is another buffer that reacts with DEPC. (Remember too, that MOPS is a much better buffer for most RNA work!)

Take-home message: **Buffers are not inert. Be careful which ones you chose.**

- **Chemical stability.** The buffer should be stable and not break down under working conditions. It should not oxidize or be affected by the system in which it is being used. Try to avoid buffers that contain participants in reactions (e.g., metabolites). Some buffers, such as MOPS, must be protected from light, but when they are stored properly they are still extremely useful buffers in biochemical reactions and laboratory protocols like RNA electrophoresis.
- **Light absorption.** The buffer should not absorb UV light at wavelengths that may be used for readouts in photometric experiments.
- **Ease of Use.** The buffer components should be easy to obtain and prepare.

Good *et al.* defined several characteristics of buffers for biochemical reactions. No matter what buffer you choose, you need to consider effects of temperature and environment on the buffer and ensure that the buffer is compatible with your system.

### III. Preparing Buffers

As discussed previously factors like temperature and concentration can greatly influence the  $pK_a$ , and therefore, the pH range over which a buffer system is most effective. Careful preparation of buffers is important for successful and reproducible experiments.

#### A. Prepare Buffers at the Appropriate Temperature and Concentration.

Because changes in temperature can be associated with a shift in dissociation, prepare your buffers at the temperature at which you will be performing your experiments. If your experiment involves a change in temperature, choose a buffer with a  $pK_a$  that accommodates it.

Changes in concentration also can be associated with a shift in dissociation, so if you plan to maintain buffer stock solutions, make sure that the pH adjustment is made after you have diluted the stock to the desired concentration and equilibrated it at the appropriate temperature. Or, at the very least, check the pH after dilution.

#### B. Adjust the pH of the Buffer System Correctly.

Many buffer materials are supplied as crystalline acids or bases (e.g., Tris base). When these materials are dissolved in water, the pH of the solution is not near the  $pK_a$ , and the pH must be adjusted using the appropriate acid or base before the solution will become a suitable buffer. If the crystalline buffer material is an acid, then pH can be

adjusted to the desired pH with a base that will not add an unwanted counter ion. If the material is a base, then an appropriate acid may be used. **Note:** Dissolve the crystalline acid or base in only 60–70% of the final desired volume to leave room for the volume of the acid or base you are using to adjust the pH. Water can be added to reach the final desired volume after the desired pH is obtained.

Many buffers, however, are not made by dissolving a crystalline acid or base then adjusting the pH to bring the solution close to the  $pK_a$ . Instead the buffer system is prepared by mixing two components, such as the free acid or base and the salt, in specific ratios to achieve the desired pH. For instance, a 0.1M solution of HEPES and a 0.1M solution of HEPES, sodium salt, can be mixed to provide a series of 0.1M HEPES buffers in a range of pH values from 6.55 to 8.55. Sodium citrate buffer solutions can be made and adjusted to the desired pH by mixing citric acid and trisodium citrate.

Other buffers are made by mixing the buffer component and its conjugate acid or base using Henderson-Hasselbalch calculations. For instance, phosphate buffers are made by mixing monobasic and dibasic sodium phosphate solutions in a specific ratio. Sodium bicarbonate buffer systems are made by mixing solutions of sodium carbonate and sodium bicarbonate.

#### C. Take Care of and Use the pH Meter Correctly.

The use of pH meters seems almost intuitive; however, pH meters must be maintained properly and electrodes cleaned and filled, and pH calibration buffers need to be correctly prepared and free of contamination. When using a pH meter, temperature is important because the pH meter electrode is temperature-dependent. The meter should be set to ambient temperature while pH is being measured. Unfortunately, the pH meter is often the most neglected piece of equipment in the laboratory. Keep a copy of the manufacturer's instructions for use readily available by the pH meter, and be sure that every laboratory member understands how to use and maintain the meter. If the pH meter is being abused, the pH of common laboratory buffers may be incorrect, and the downstream consequences could be disastrous.

#### D. Random Tips about Buffer Preparation

- Check all stored buffers before use; if they look cloudy or discolored, do not use them. Such solutions may have microbial contamination or may have become chemically unstable. One exception to this is MOPS, which sometimes appears slightly yellow. When checking for signs of contamination, be sure to swirl the bottle because contaminants can settle to the bottom.
- When creating a set of instructions for laboratory buffer preparation, be complete because good science depends on being able to replicate experiments, and experiments cannot be replicated if the buffers are not made correctly and consistently. Be sure to include grades of materials used, sources, etc. Indicate what acid or base was used and the recommended concentration. Also, be sure to

state at what point during the preparation pH measurements were made; this is especially important if you are adding additional components to the buffer.

- Some buffer materials (e.g., PIPES) do not go into solution easily and require a slightly alkaline or acid environment or heating before they will dissolve.
- Some buffers (e.g., MOPS and HEPES) cannot be autoclaved because they degrade upon heating.
- Buffers containing primary amines, like Tris and glycine, interfere with the Bradford dye-binding protein assay (Stoll and Blanchard, 1990).
- When working with acids and bases, wear protective clothing and eyewear. Do not neutralize a strong acid with a strong base, because this generates an exothermic reaction that can melt the container you are using, for example.
- If for some reason you are using a solvent other than water, be sure you know how that affects the  $K_a$  of your buffering agent.

---

#### Online Resources for Preparing Buffers

##### Online Tools

[Molarity Calculator](#)

##### Online Tools

[Dilution Calculator](#)

##### Online Tools

[Buffer Calculator from the University of Liverpool](#)

---

#### IV. Appendix A: The Henderson-Hasselbalch Equation

Using acetic acid as an example, the equilibrium relationship of a weak acid, hydrogen ion and the conjugate base can be expressed mathematically as:

$\text{HAc} \rightleftharpoons \text{H}^+ + \text{A}^-$ , where the rate constant of dissociation of acetic acid is  $k_1$  and the rate constant of association of acetate and hydrogen ion is  $k_2$ . The rate of dissociation of acetic acid ( $-d[\text{HAc}]/dt$ ) depends on the rate constant of dissociation and the concentration of acetic acid and can be written as:

$$-d[\text{HAc}]/dt = k_1 [\text{HAc}]$$

Likewise the rate of association of acetate ion and hydrogen ion to form acetic acid ( $d[\text{HAc}]/dt$ ) also depends on the rate constant of association ( $k_2$ ) and the concentration of acetate and hydrogen ions:

$$d[\text{HAc}]/dt = k_2 [\text{H}^+][\text{A}^-]$$

At equilibrium, the rates of association and dissociation are equal, so

$$k_1 [\text{HAc}] = k_2 [\text{H}^+][\text{A}^-] \text{ or } k_1/k_2 = [\text{H}^+][\text{A}^-]/[\text{HAc}]$$

and

$$K_a \text{ (the equilibrium constant)} = [\text{H}^+][\text{A}^-]/[\text{HAc}], \text{ where } k_1/k_2 = K_a$$

We can rearrange that equation to express hydrogen ion concentration in terms of the equilibrium constant and the undissociated acetic acid and acetate ion.

$$[\text{H}^+] = K_a ([\text{HAc}]/[\text{A}^-])$$

Since  $\text{pH} = -\log [\text{H}^+]$  and  $\text{p}K_a$  is defined as  $-\log K_a$ , we can convert the equilibrium expression above to  $-\log$ :

$$-\log [\text{H}^+] = -\log K_a - \log ([\text{HAc}]/[\text{A}^-])$$

Substituting, pH and  $\text{p}K_a$  at the appropriate points:

$$\text{pH} = \text{p}K_a - \log ([\text{HAc}]/[\text{A}^-])$$

To change the sign of the  $-\log$ , invert the  $[\text{HAc}]/[\text{A}^-]$ :

$$\text{pH} = \text{p}K_a + \log ([\text{A}^-]/[\text{HAc}])$$

and you have the Henderson-Hasselbalch equation. Using this equation, you can calculate pH when concentrations of acid and base and  $\text{p}K_a$  are known. The  $\text{p}K_a$  for a buffer system determines the pH range at which that buffer is most effective.

**V. Appendix B: Composition and Preparation of Common Buffers and Solutions****A. Preparation of Bicarbonate-Carbonate Buffer (pH 9.2–10.8)**

To create 100ml of a 0.1M bicarbonate buffer solution, mix sodium bicarbonate and sodium carbonate, decahydrate, as given below.

Solution A: 0.1M sodium bicarbonate ( $\text{NaHCO}_3$  MW = 84.0) (MW = molecular weight)

Solution B: 0.1M sodium carbonate, decahydrate ( $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$  FW = 286.2) (FW = formula weight)

**Table 15.1. Bicarbonate-Carbonate Buffer.**

pH at 20°C	pH at 37°C	Solution A (ml)	Solution B (ml)
9.4	9.1	80	20
9.5	9.4	70	30
9.8	9.5	60	40
9.9	9.7	50	50
10.1	9.9	40	60
10.3	10.1	30	70
10.5	10.3	20	80
10.8	10.6	10	90

**B. Preparation of Citrate Buffer (pH 3.0–6.2)**

To create 100ml of a 0.1M citrate buffer, mix citric acid, monohydrate, and trisodium citrate dehydrate as given below.

Solution A: 0.1M citric acid monohydrate ( $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$  FW = 210.14)

Solution B: 0.1M trisodium citrate, dihydrate ( $\text{C}_6\text{H}_5\text{O}_7\text{Na}_3 \cdot 2\text{H}_2\text{O}$  FW = 294.12)

**Table 15.2. Citrate Buffer.**

pH	Solution A (ml)	Solution B (ml)
3.0	82.0	18.0
3.2	77.5	22.5
3.4	73.0	27.0
3.6	68.5	31.5
3.8	63.5	36.5
4.0	59.0	41.0
4.2	54.0	46.0
4.4	49.5	50.5
4.6	44.5	55.5
4.8	40.0	60.0
5.0	35.0	65.0
5.2	30.5	69.5
5.4	25.5	74.5
5.6	21.0	79.0
5.8	16.0	84.0
6.0	11.5	88.5
6.2	8.0	92.0

**C. Preparation of Phosphate Buffer (pH 5.8–8.0 at 25°C)**

To create 100ml of a 0.1M phosphate buffer, mix sodium phosphate, dibasic dihydrate and sodium phosphate monobasic monohydrate, as given below, and dilute to 100ml with water.

**Note:** The dibasic stock sodium phosphate may be somewhat harder to dissolve; adding a little heat may help.

Solution A: 0.2M sodium phosphate, dibasic dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  FW = 178.05)

Solution B: 0.2M sodium phosphate, monobasic, monohydrate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  FW = 138.01)

**Table 15.3. Phosphate Buffer.**

pH at 25°C	Solution A (ml)	Solution B (ml)
5.8	4.0	46.0
6.0	6.15	43.85
6.2	9.25	40.75
6.4	13.25	36.75
6.6	18.75	31.25
6.8	24.5	25.5
7.0	30.5	19.5
7.2	36	14
7.4	40.5	9.5
7.6	43.5	6.5
7.8	45.75	4.25
8.0	47.35	2.65

**D. Composition of Additional Buffers and Solutions****1M HEPES (pH 7.5)**

- 23.83g HEPES
- Water to 100ml

Adjust to pH 7.5 with potassium hydroxide (KOH). Store at 4°C.

**5X MOPS buffer**

- 0.2M MOPS (pH 7.0)
- 0.05 sodium acetate
- 0.005M EDTA (pH8.0)

For 2 liters of buffer, add 83.72g of MOPS (free acid) and 8.23g of sodium acetate to 1.6 liters of DEPC-treated water and stir until completely dissolved. Add 20ml of DEPC-treated 0.5M EDTA, and adjust the pH to 7.0 with 10N NaOH. Bring the final volume to 2 liters with DEPC-treated water. Filter sterilize and dispense into aliquots.

**phosphate-buffered saline (PBS)**

- 8g NaCl, 0.2g KCl
- 1.44g  $\text{Na}_2\text{HPO}_4$
- 0.24g  $\text{KH}_2\text{PO}_4$

Dissolve salts in 800ml of distilled water. Adjust to pH 7.4 with HCl. Add water to 1 liter. Dispense into aliquots. Sterilize by autoclaving.

**PBS (Mg<sup>2+</sup>- and Ca<sup>2+</sup>-free)**

- 137mM NaCl
- 2.7mM KCl
- 4.3mM Na<sub>2</sub>HPO<sub>4</sub>
- 1.4mM KH<sub>2</sub>PO<sub>4</sub>

The final pH should be 7.4 at 25°C.

**PBST (pH 7.4)**

- 137mM NaCl
- 2.7mM KCl
- 8.1mM Na<sub>2</sub>HPO<sub>4</sub>
- 1.47mM KH<sub>2</sub>PO<sub>4</sub>
- 0.05–1% Tween® 20

**50X TAE**

Dissolve 242g of Tris base and 37.2g of Na<sub>2</sub>EDTA•(2H<sub>2</sub>O) in 900ml of deionized water. Add 57.1ml of glacial acetic acid, and adjust the final volume with water to 1 liter. Store at room temperature or 4°C.

**10X TBE**

Dissolve 108g of Tris base and 55g of boric acid in 900ml of deionized water. Add 40ml of 0.5M EDTA (pH 8.0), and adjust the final volume with water to 1 liter. Store at room temperature or 4°C.

**TBST**

- 20mM Tris-HCl (pH 7.5)
- 150mM NaCl
- 0.05–0.1% Tween® 20

**TE buffer**

- 10mM Tris-HCl (pH 8.0)
- 1mM EDTA

**TEN buffer**

- 40mM Tris-HCl (pH 7.5)
- 1mM EDTA (pH 8.0)
- 150mM NaCl

**VI. References**

Arduengo, P.M. (2010) Sloppy technicians and the progress of science. *Promega Connections*

<http://promega.wordpress.com/2010/03/15/sloppy-technicians>

Good, N.E. *et al.* (1966) Hydrogen ion buffers for biological research. *Biochemistry*, **5**, 467–77.

Stoll, V.S. and Blanchard, J.S. (1990) Buffers: Principles and practice. *Meth. Enzymol.* **182**, 24–38.

Tween is a registered trademark of ICI Americas, Inc.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

© 2004–2012 Promega Corporation. All Rights Reserved.