Buffered Ethanol Fixative

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John A. Kiernan

Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada (JAK)

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In the recent *Journal of Histochemistry & Cytochemistry* (JHC) article by Perry et al.,¹ a fixative mixture designated “buffered ethanol 70%” (BE70) was judged best for preserving structural integrity, providing satisfactory immunostaining with three commonly used antibodies, and for allowing optimal extraction and amplification of nucleic acids. In the abstract, BE70 is called “phosphate buffered ethanol 70%.” The designation “buffered ethanol 70% (BE70)” is used also in the legends of Figures 1, 2, and 3. In the “Methods” section of the article, “70% ethanol + 0.5× phosphate-buffered saline . . . (EP)” most accurately corresponds to “phosphate buffered ethanol 70%,” but BE70 is identified as “70% ethanol + 1% glycerol + 0.5% glacial acetic acid + 0.5× PBS (EGAP; BE70).” The article does not provide enough information to allow other investigators to make the mixture that the authors favored for combined histological and biochemical preservation. If BE70 is a mixture in which acetic acid and a phosphate-buffered aqueous salt solution were mixed with ethanol, what is its approximate pH? This information is needed to define the composition of the BE70 fixative.

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Corresponding Author:

John A. Kiernan, Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada N6A 5C1.
E-mail: jkiernan@uwo.ca

Formulation and pH of the Buffered Ethanol Fixative BE70

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Stephen M. Hewitt

National Cancer Institute, National Institutes of Health, Bethesda, Maryland (SMH)

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In response to the letter to the editor by Kiernan¹ concerning details on formulation and pH of BE70,² we apologize for the inconsistent use of abbreviations in the formulation of the fixative. Our initial discovery was that a fixative of 70% ethanol made with (1×) PBS was superior to a fixative of 70% ethanol made with distilled water. This initiated a series of studies, of which only a limited number of combinations could be accommodated in the final article, resulting in the final fixative we termed BE70.

The notation “(EGAP; BE70)” would have been better left as only “BE70.” “EGAP” is the internal laboratory shorthand of the components—ethanol, glycerol, (glacial acetic) acid, and P(BS). The formulation of BE70 is as stated:

70% ethanol, absolute
1% glycerol
0.5% glacial acetic acid
28.5% 0.5× PBS.

This rather arcane approach to describing the formulation of solutions is in fact common to many

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Corresponding Author:

Stephen M. Hewitt, Experimental Pathology Laboratory, Laboratory of Pathology Center for Cancer Research, National Cancer Institute, National Institutes of Health, 10 Center Drive, Bethesda, MD 20892, USA.
E-mail: genejock@helix.nih.gov

histology manuals. A more standard recipe for BE70 is as follows.

First prepare stock solution consisting of 50 ml of 10× PBS (pH 7.4), 12.5 ml of 80% glycerol, and 5 ml of glacial acetic acid. Adjust the pH to 4.3 with NaOH and then fill to 300 ml with distilled water. Before use, mix 300 ml of this stock solution and 700 ml of absolute (200 proof) EtOH for a total volume of 1000 ml. The pH of the final composition is approximately 6.1, and the fixative has no precipitate at 4°C storage.

In reference to the pH of BE70, we evaluated BE70 at different pH values ranging from 4 to 7, and were unable to discern a measurable difference in histomorphology or RNA quality, which we have previously demonstrated to be the most sensitive metric for tissue fixation and processing³; however, we chose a more neutral pH in an effort to avoid requiring additional shipping and storage precautions associated with acid pH reagents, as well as not damage tissue processing hardware. The subject of fixative pH is complex. To our knowledge, the first report concerning the introduction of buffers into a fixative is from 1894 by Mann,⁴ as recounted by Burke,⁵ who argued for pyridine–formalin. Lillie comments on the common use of calcium carbonate and magnesium carbonate as buffering agents, resulting in pH levels between 6.3 and 7.5.⁶ Fox

notes that a pH above 6.1 reduces the formation of “formalin pigment,” a derivative of hematin.⁷ We did not observe the formation of any pigments associated with pH.

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