

Short Technical Reports

Fixation and Decalcification of Adult Zebrafish for Histological, Immunocytochemical, and Genotypic Analysis

BioTechniques 32:296-298 (February 2002)

ABSTRACT

To facilitate the molecular analysis of tissues in adult zebrafish, we tested eight different fixation and decalcification conditions for the ability to yield DNA suitable for PCR and tissue immunoreactivity, following paraffin embedding and sectioning. Although all conditions resulted in good tissue histology and immunocytochemistry, only two conditions left the DNA intact as seen by PCR. The results indicate that zebrafish fixed in either 10% neutral buffered formalin or 4% paraformaldehyde, followed by decalcification in 0.5 M EDTA, is an easy and reliable method that allows molecular experiments and histology to be performed on the same specimen. The fixation and decalcification by Dietrich's solution permitted the PCR amplification of DNA fragments of 250 but not 1000 bp. Therefore, a protocol of formalin or paraformaldehyde fixation followed by decalcification with EDTA is broadly applicable to a variety of vertebrate tissues when excellent histological, immunocytochemical, and genotypic analyses may be simultaneously required.

INTRODUCTION

The zebrafish is an increasingly popular model system for studying the molecular basis of a variety of biological phenomena. The examination of preserved tissue from adult or juvenile fish can be an important component of these experiments. However, whole zebrafish, like any tissue containing bone, must be decalcified before sectioning. Most solutions used for decalcification involve acidic conditions that may damage DNA. As part of our ongoing research of cancer in zebrafish, we asked what combination of available methods would allow us to fix and decalcify adult zebrafish tissues for rou-

tine histology, immunohistochemistry, and PCR-based genotypic analyses of microdissected tissue samples. We tested two different formaldehyde fixatives in combination with three different decalcification solutions. Two commonly used, acid-based fixatives that simultaneously fix and decalcify, Bouin's and Dietrich's solutions (1,4), were also tested. The goal of these experiments was to determine whether high-quality histological specimens could be obtained from decalcified tissues without the degradation of DNA.

MATERIALS AND METHODS

Two adult zebrafish were treated according to each of the eight conditions listed in Table 1. The fish were 13-month-old male siblings of a laboratory-raised, wild-type stock, originally obtained from a Florida fish farm (Lyles, Ruskin, FL, USA). Fish were anesthetized in an excess of tricaine methanesulfonate (MS222; Argent, Redmond, WA, USA). The belly was then opened along the ventral midline from the anal pore to just below the gills to allow solutions into the body cavity. Both fixation and decalcification were done with an excess of fluid, approximately 100 mL for two fish, for a solution to tissue ratio of approximately 60:1. All fish were fixed and decalcified at room temperature, approximately 25°C, except for fish fixed in 4% paraformaldehyde, which were incubated at 4°C. Fish were rinsed twice with Dulbecco's PBS (Invitrogen, Carlsbad, CA, USA) before and after decalcification. A portion of the tail fin was clipped from each fish before fixation for DNA extraction, and a second fin clip was taken following fixation and decalcification but before embedding. Fixation solutions included 4% freshly prepared paraformaldehyde, pH 7.2–7.4, 10% neutral buffered formalin, pH 7.0, Bouin's solution (75% picric acid, 10% formalin, and 5% acetic acid, pH 1.0), and Dietrich's solution (30% ethanol, 10% formalin, and 2% acetic acid, pH 2.7) (1,3,4). Decalcification solutions included 1.35 M HCl Cal-EX® (Fisher Scientific, Pittsburgh, PA, USA), 10% sodium citrate/22.5% formic acid, pH 2.5, and 0.5 M EDTA, pH 7.8.

Paraffin embedding, sectioning, and staining with regressive Harris hematoxylin and eosin were done according to standard protocols (1,4). To obtain DNA samples after sectioning, slides were deparaffinized by immersion in xylene, 100% ethanol, 95% ethanol (2 incubations of 1 min each), and then air dried (6). A 25-gauge needle was used to microdissect 1–2 mm² regions of tissue into 100 µL DNA extraction buffer (7). DNA from zebrafish tail fin clips was also prepared using a standard protocol (2). Paraffin sections from each condition were deparaffinized and tested for immunoreactivity with antibodies to muscle-specific actin (1:800 dilution; Biomeda Immunochemicals, Foster City, CA, USA) and cytokeratin (undiluted anti-cytokeratin AE1, 3; Roche Molecular Biochemicals, Indianapolis, IN, USA). Reactions were done using a Vectastain® Elite ABC Kit, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA), with amino-ethyl-carbazole as the chromogen and hematoxylin as the counterstain.

PCR was performed using 5 µL DNA in a 20 µL reaction containing 0.2 µM each primer and amplified for 35–40 cycles using *Taq* DNA polymerase (Fisher Scientific) as previously described (2). Fragments of approximately 250 bp were amplified using SSR Z13836 (GenBank® accession no. G40079) and primers from the zebrafish Na, K-ATPase α subunit Clone 3 (accession no. AF286374) (5). A fragment of 1000 bp was amplified from the coding sequence of the zebrafish Na, K-ATPase α subunit Clone 2 (accession no. AF286373) (5).

RESULTS AND DISCUSSION

All of the eight conditions for fixation and decalcification of adult zebrafish resulted in high-quality paraffin sections for hematoxylin and eosin staining and immunohistochemistry (Figure 1). However, formaldehyde fixation followed by decalcification in EDTA (4) best preserved the DNA for PCR (Table 1 and Figure 2a). With 10% neutral buffered formalin or 4% paraformaldehyde as a fixative, in combination with 0.5 M EDTA decalcification,

Table 1. Fixation and Decalcification Conditions Tested and Summary of PCR Results

Condition	Fixation	Decalcification	Total Incubation	250-bp ^a Product	1000-bp Product
1	NBF, 24 h	Cal-EX, 1 h, 15 min	25 h, 15 min	No	No
2	NBF, 24 h	Sodium Citrate/Formic Acid, 24 h	2 days	No	No
3	NBF, 24 h	0.5 M EDTA, 7 days	8 days	Yes	Yes
4	4% PFA, 24 h, (4°C)	Cal-EX, 1 h, 15 min	25 h, 15 min	No	No
5	4% PFA, 24 h, (4°C)	Sodium Citrate/Formic Acid, 24 h	2 days	No	No
6	4% PFA, 24 h, (4°C)	0.5 M EDTA, 7 days	8 days	Yes	Yes
7	Bouin's Solution		24 h	No	No
8	Dietrich's Solution		3 days	Yes, faint	No

NBF, neutral buffered formalin; PFA, paraformaldehyde
^aTwo different primer sets were used on all samples to obtain the data summarized here.

we were able to reliably amplify 250- and 1000-bp DNA fragments. Dietrich's fixative (Table 1, Condition 8) is a mixture of ethanol, formalin, and acetic acid (1) that has both fixation and decalcification properties and is recommended by the Zebrafish International Resource Center to preserve zebrafish for histopathological examination (http://zfin.org/zf_info/stckctr/fix.html). DNA from tissues treated with Dietrich's solution

yielded amplification of 250-bp DNA fragments. However, the reliability of the PCR amplification of DNA obtained from fish decalcified with Dietrich's solution was less than that obtained from fish decalcified using EDTA (Conditions 3 and 6). Amplification of a 1000-bp DNA fragment from Dietrich's decalcified tissue was unsuccessful. Cal-EX, a hydrochloric acid solution, produced the most rapid decalcification

but caused enough DNA damage to prevent the formation of any PCR products (Conditions 1 and 4). The sodium citrate/formic acid decalcification also appeared to destroy DNA since no PCR products were seen from fish decalcified with this solution (Conditions 2 and 4). Bouin's solution (Condition 7), a mixture of picric acid, formalin, and acetic acid, is a popular combined fixative/decalcification solution that acts rapidly and provides excellent histology (1,4). However, no amplifiable DNA was obtained from tissues treated with Bouin's fixative, indicating that it is not a good choice for subsequent genotypic analysis. Cal-EX, sodium citrate/formic acid, and Bouin's solution are all acidic solutions that are effective for decalcification but will also depurinate nucleic acids, which explains the lack of PCR products from tissue treated with these solutions. While Dietrich's solution also has an acid component, the acetic acid concentration in it is apparently mild enough so that some DNA remains. On the other hand, EDTA can be prepared to a physiologic pH of 7.0–8.0 and, therefore, does little or no harm to nucleic acids. The only disadvantage of using EDTA for decalcification is that it requires a longer incubation than the acidic solutions.

As an example of the potential power of the technique described here, DNA was prepared from a variety of organs from fish that were fixed in either neutral buffered formalin or paraformaldehyde, followed by decalcification in EDTA (Conditions 3 and

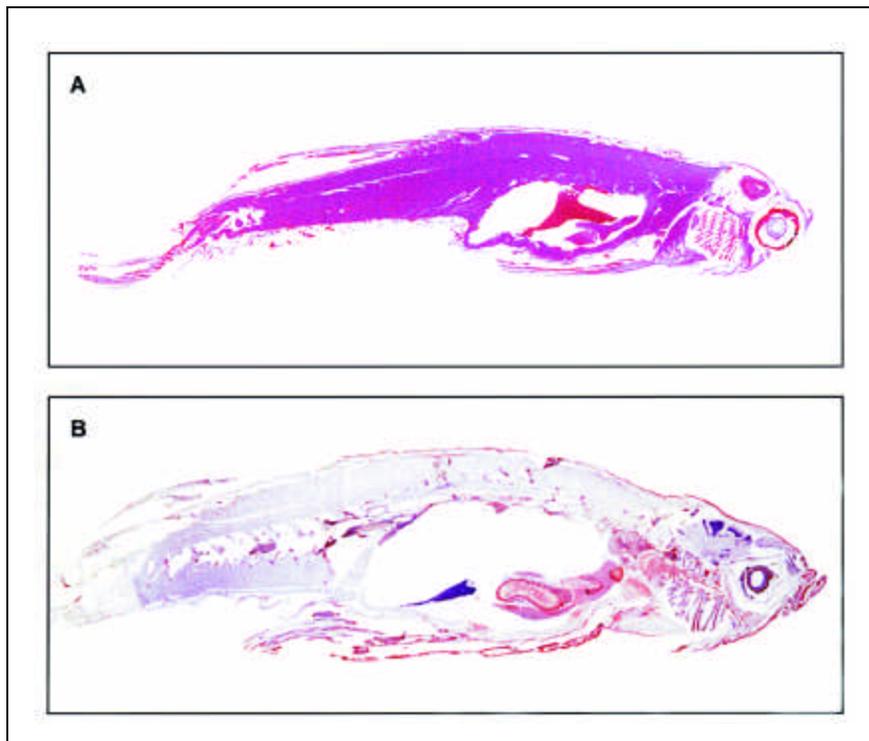


Figure 1. Paraffin-sectioned zebrafish fixed in 4% paraformaldehyde and decalcified in 0.5 M EDTA. (A) Hematoxylin and eosin stain and (B) anti-cytokeratin (red) and hematoxylin (blue).

Short Technical Reports

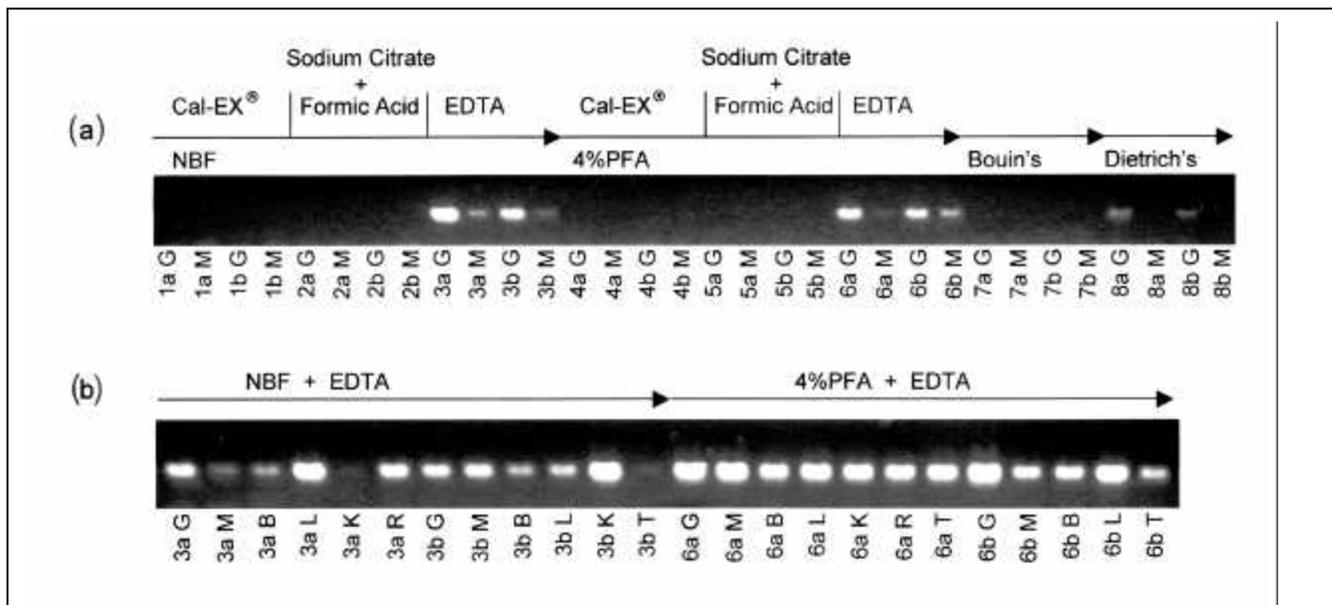


Figure 2. PCR amplification of a 250-bp DNA fragment from zebrafish fixed and decalcified in various conditions. (a) Attempted amplification of DNA from zebrafish from all fixation and decalcification conditions. (b) Amplification of DNA from multiple tissues of formalin- or paraformaldehyde-fixed, EDTA-decalcified zebrafish. G, gill; M, muscle; B, brain; L, liver; K, kidney; R, retina; and T, testes. Samples were scraped from deparaffinized sections from two fish (a and b) from each experimental condition as indicated.

6) using the corresponding hematoxylin and eosin-stained slides as a reference. Figure 2b shows the PCR results from DNA obtained from microdissected gill, muscle, brain, liver, kidney, retina, and testes of these samples. These tissues amplified with variable intensity because of both the nuclear density of the particular organ and the amount of the organ present in the section. The slides from which these tissues are isolated can be later stained with hematoxylin and eosin for reference (data not shown).

In conclusion, we have shown that it is possible to prepare an entire adult zebrafish for histology and immunohistochemistry in such a way that DNA can also be obtained for genotypic analysis. Of the methods tested here, zebrafish fixed in 10% neutral buffered formalin or 4% paraformaldehyde for 24 h, followed by decalcification for 5–7 days in 0.5 M EDTA permitted histological, immunocytochemical, and genotypic analyses from the same tissues. In work to be reported elsewhere, similar methods can also be used to preserve the fluorescence of GFP in fixed, decalcified, and sectioned mouse skeletons (Harms and Welch, personal communication). Together the results suggest that these methods may be broadly applicable to

any vertebrate model system in which simultaneous histological, immunocytochemical, genotypic, and fluorescent analyses are desired.

REFERENCES

1. Fournie, J.W., R.M. Krol, and W.E. Hawkins. 2000. Fixation of fish tissues, p. 569-578. In G.K. Ostrander (Ed.), *The Laboratory Fish*. Academic Press, New York.
2. Gestl, E.E., E.J. Kauffman, J.L. Moore, and K.C. Cheng. 1997. New conditions for generation of gynogenetic half-tetrad embryos in zebrafish (*Danio rerio*). *J. Heredity* 88:76-79.
3. Greer, C.E., C.M. Wheeler, and M.M. Manos. 1995. PCR Amplification from paraffin-embedded tissues: sample preparation and the effects of fixation, p. 99-112. In C.W. DiEffenbach and G.S. Dveksler (Eds.), *PCR Primer: A Laboratory Manual*. CSH Laboratory Press, Cold Spring Harbor, NY.
4. Luna, L.G. (Ed.) 1960. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. McGraw Hill, New York.
5. Rajarao, S.J.R., V.A. Canfield, M.P.K. Mohideen, Y.L. Yan, J.H. Postlethwait, K.C. Cheng, and R. Levenson. 2001. The repertoire of Na, K-ATPase α and β subunit genes expressed in the zebrafish, *Danio rerio*. *Genome Res.* 11:1211-1220.
6. Thibodeau, S.N., G. Bren, and D. Schaid. 1993. Microsatellite instability in cancer of the proximal colon. *Science* 260:816-819.
7. Tsao-Wu, G.S., C.H. Weber, L.R. Budgeon, and K.C. Cheng. 1998. Agarose-embedded tissue arrays for histologic and genetic analysis. *BioTechniques* 25:614-618.

The authors thank Lynn Budgeon and Xiao-Hong Wang for advice, assistance, and technical help in preparing the histology and immunocytochemistry samples. We also thank Joe Rajarao and Robert Levenson for the gift of PCR primers to the zebrafish Na, K-ATPase gene and Dr. Andrew Wong for assistance with PCR. This work was supported by the Jake Gittlen Memorial Golf Tournament, National Institutes of Health grant no. RO1-CA73935 to K.C.C. and NRSA-F32-GM119794 to J.L.M., and National Science Foundation grant no. MCB-93198174 to K.C.C. Address correspondence to Dr. Keith C. Cheng, The Jake Gittlen Cancer Research Institute, H059, Pennsylvania State College of Medicine, 500 University Drive, Hershey, PA 17033, USA. e-mail: kcheng@psu.edu

Received 8 May 2001; accepted 31 August 2001.

Jessica L. Moore, Michele Aros, Kimberly G. Steudel, and Keith C. Cheng
Pennsylvania State College
of Medicine
Hershey, PA, USA