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A Simple Procedure for Long-Term Storage and Preparation of Fish Cells for DNA Content Analysis Using Flow Cytometry

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Abstract.—A simple procedure for long-term storage and preparation of fish cells for flow cytometric analysis of genome size or DNA content is described. The method has been used on a broad variety of cell or tissue types, including blood, soft organs, and muscle, and on fish ranging in size from 10 to 15 mm to over 500 mm in total length. No differences in estimated genome sizes or coefficients of variation have been observed with storage times of up to 8 months.

In the last few years, there has been a renewed interest in measuring the genome size or DNA content of individual fish. Several researchers (Gold and Amemiya 1987; Johnson et al. 1987; Tiersch et al., in press) have focused on questions related to either the evolution of genome size in different fish groups or the variation in genome size in wild or domesticated fish populations. Others (Thorgaard et al. 1982; Allen 1983) have used DNA content analysis to verify ploidy levels following shock treatments of eggs or embryos. The latter is of particular interest to programs in both aquaculture and fisheries management for reasons listed in Thorgaard (1983) and Thorgaard and Allen (1987).

A variety of methods, including chemical analysis (Hudson 1976), scanning microdensitometry (Gold and Amemiya 1987; Ragland and Gold 1989), and flow cytometry (Thorgaard et al. 1982; Johnson et al. 1987; Tiersch et al., in press), have been used to assay genome size. Of these, flow cytometry is by far the most preferred in terms of precision and reproducibility (Muirhead et al. 1985; Shapiro 1985). A critical need in flow cytometric analysis of fish material is a reliable procedure for long-term storage of samples. Frequently, individuals to be assayed are obtained at some distance from the flow cytometer to be used and cannot easily be transported alive to the laboratory. In addition, it is often necessary to process numerous individuals at the same time (or developmental stage), even though, for several reasons, the samples cannot be analyzed quickly. One common reason for such delays is that flow cytometers are expensive, and typically there are many users of a single cytometer.

A simple procedure for long-term storage and preparation of fish cells for genome size analysis using flow cytometry is described here. Our procedure was modified from that described by Vindelov et al. (1982) and has been used primarily on freshwater fish species. The method appears to be applicable to a broad variety of cell or tissue types and can be used on fish as small as 10 mm in length. In addition to sample collection and storage, preparation and staining conditions for flow cytometry are also described.

The preferred sample material for flow cytometric analysis of fish genome size is whole blood because it can easily be obtained from individuals at least 40 mm long. We usually obtain blood samples by inserting a heparinized microcapillary tube directly into the heart and drawing or teasing the blood into the tube. For specimens longer than 500 mm, puncture of the caudal vein may also be employed. Up to 40 μL of blood can be obtained in one microcapillary tube. The blood is added to a cryogenic storage tube (cryovial) containing an equal volume of storage buffer and then immediately placed in liquid nitrogen. The storage buffer is made in 1-L quantities as follows: 85.5 g sucrose (250 mM), 11.76 g trisodium citrate-dihy-

drate (40 mM), and 50 mL dimethyl sulfoxide (DMSO) in 1 L of distilled water brought to pH 7.60 with HCl.

Internal organs (primarily kidney, heart, and liver) may also be used for flow cytometric analysis of genome size by removing the organs and placing them in the cryovials. Care should be taken not to include the gut because the latter often contains parasitic organisms that can interfere with genome size determinations. Approximately 5 mm^3 of tissue are placed into each cryovial with enough storage buffer (approximately 80 μL) to completely immerse the sample. The cryovial is then immediately placed in liquid nitrogen. On very small specimens of 10 mm or so, muscle tissue may be used instead of soft organs. For any of the tissues, samples are kept in liquid nitrogen for transport to the laboratory and then stored in an ultracold (−80°C) freezer.

Samples are prepared for flow cytometry by warming the cryovials to room temperature. For blood, subsamples (10 μL) are withdrawn from each cryovial and dounced 6–10 times in a 2-mL Kontes dounce with 0.6 mL of 75% sodium citrate. If the blood-to-storage buffer ratio is less than one, the volume of subsample should be increased accordingly (up to 40 μL). For soft organs or muscle, the tissue is removed from the cryovial and dounced up to 20 times with the same size dounce and volume of 75% sodium citrate as for blood. Dounced subsamples are filtered through a 41-μm nylon filter (Macro-filter N by Spectra) into a microcentrifuge tube and centrifuged for 3–8 s in a microcentrifuge. The supernatant is discarded and the pellet resuspended in 0.3–0.5 mL of staining solution by vortexing briefly. The staining solution is made in 100-mL quantities as follows: 0.8 g sodium chloride (137 mM), 0.056 g PIPES, 0.028 g disodium EDTA (0.75 mM), 4 mg of deoxyribonuclease-free (boiled for 5 min) ribonuclease, and 5 mg propidium iodide in 100 mL of distilled water brought to pH 7.50 with NaOH. The centrifugation and resuspension steps are repeated twice. The final suspension is then held on ice or in the refrigerator at 4°C for 20 min to ensure thorough staining of the nuclei. Flow cytometric standards (erythrocytes from chicken, *Gallus gallus,* or koi, *Carassius auratus*) prepared in the same way are then added to the sample.

For flow cytometry, we use an Ortho Cytofluorograf 50H equipped with an 8W Lexel Model 95 ion laser, a flow cell assay system, and an Ortho 2140 data acquisition system. The laser is set at the 514-nm wavelength with 1.8 W of output. Two signals are used for selecting and recording histogram data. A 90° red fluorescence signal is used to trigger counts in the area integration mode for measuring DNA. Forward scatter of green light is used to select clean nuclei from other cellular debris. The photomultipliers and the flow cell are adjusted for optimum output with the chicken or koi standards (or both). Gates are set on the 90° red histogram at the inflection points in the data peaks. The gate on the 90° versus forward scatter histogram is set at the beginning of the run (or stored in memory as part of the histogram) and remains constant.

A sample histogram from soft organ tissue of largemouth bass (*Micropterus salmoides*) is shown in Figure 1, and comparisons of genome size estimates and coefficients of variation from samples stored for varying periods of time are shown in Table 1. To date, we have examined genome sizes of over 250 individuals from four families of fish-

TABLE 1.—Genome size data from flow cytometric analysis of samples stored for varying periods of time. Data are shown as ranges of values from three specimens per storage time and size-class.

Species	Storage time (months)	Total length (mm)	Cells counted	Genome size[a] (pg)	Coefficient of variation (%)
Largemouth bass	1	15–50	200–400	1.96–2.02	2.0–2.5
	2	15–50	500–1,800	1.93–2.02	3.9–4.8
	3	15–50	350–800	1.97–2.03	2.9–3.9
	6	15–50	600–950	1.96–2.01	3.4–3.6
	1	100–150	1,800–3,800	1.95–2.00	2.5–2.8
	8	250–300	1,000–1,400	1.97–2.02	2.4–2.6
Longear sunfish[b]	8	75–100	5,200–5,800	2.10–2.15	2.4–3.7
Bluegill[c]	4	40–75	750–3,400	1.93–1.99	2.3–2.9

[a] Genome size data are in picograms of DNA. Genome size values observed fall well within the range of genome sizes reported for each of the three species (Ragland 1986; Ragland and Gold 1989).

[b] *Lepomis megalotis.*

[c] *Lepomis macrochirus.*

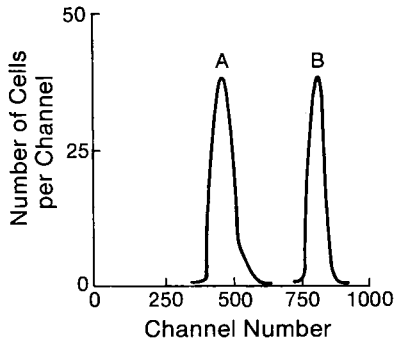

FIGURE 1.—DNA flow histogram of largemouth bass tissue (peak A) and a koi standard (peak B). The number of cells counted under peak A was 1,615; the mean channel number and coefficient of variation were 452 and 3.6%, respectively. The number of cells counted under peak B was 1,756; the mean channel number and coefficient of variation were 814.6 and 2.1%. If the genome size of koi is assumed to be 3.69 pg of DNA, the genome size of the largemouth bass is estimated to be 2.05 pg of DNA.

es. The lengths of the individuals assayed have ranged from about 10 mm to over 500 mm. Average coefficients of variation have ranged from 2 to 5% regardless of tissue type and over storage times of up to 8 months. The simplicity of the method and its reproducibility in terms of results suggest it will be broadly applicable in situations where long-term storage of material for flow cytometric analysis is needed.

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