Protocol K - Erythrocyte measurements [Pratical guide of protocols: methods of verification of the ploidy]

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I - Introduction

As triploid fish exhibit 50% more DNA per cell, total cell volume in triploids increase significantly in comparison to that of diploids to accommodate the extra amount of chromosomes. In particular, erythrocyte cellular and nuclear measurements are proportional to ploidy in teleost fish. On this basis, and assuming that erythrocytes in fish show an ellipsoidal shape, erythrocyte cellular and nuclear volume have been used as the criterion to identify polyploid fish in several studies (Wolters *et al*., 1982; Benfey *et al*., 1984; Thititananuki *et al*., 1996; Felip *et al*., 2001a; Cal *et al*., 2005).

Thus, triploid is estimated from blood smears without slaughtering the fish. As previously demonstrated in other fish species, studies in our laboratory have also supported that triploid sea bass can be accurately identified solely on the measure of the major axis either the cell or the nucleus and even, on the measure of the minor axis (Felip *et al.*, 1997; Felip *et al*., 2001a). Thus, the use of erythrocyte measurements is considered an indirect approach to identify triploids that does not require special equipment or expertise, making it valuable for its use in the field and the commercial facilities. Nevertheless, the accuracy of this methodology has been in question for the identification of mosaic polyploids and the occurrence of misclassification when the ranges of erythrocyte measurements overlap. Consequently, the validity of erythrocyte measurements to determine ploidy levels for a particular species must be previously verified.

Correlation and accuracy analyses between erythrocyte measurements and cytogenetically determined ploidy levels must be confirmed before erythrocyte measurements can be used as a reliable estimation of ploidy level. Many studies support this technique as a viable alternative to karyotyping and its use has been extended for the identification of triploids in both freshwater and marine teleost species (Ihssen *et al*., 1990; Felip *et al.*, 2001b).

II - Purpose

The objective of this Protocol is to report the accuracy of using erythrocyte cellular and nuclear measurements to identify triploid sea bass. The procedure of this technique shows that it is a routinely and non-invasive approach for an easy, fast and inexpensive use in the laboratory.

III - Procedure

(i) Extract a few microlitres of blood and extend it on a precleaned micro slide (see Fig. K.1).

(ii) Allow blood smear to air dry for 10-15 minutes and fix it with ethanol. If blood smear is immediately stained, fixation is not necessary.

(iii) Stain blood smear with 15% Giemsa for 45 minutes.

(iv) Wash blood smear with distilled water and allow it to air dry for 10-15 minutes.

(v) Wash it with xylen for 10 minutes.

(vi) Add a cover glass using 2-3 drops of DPX.

(vii) Use a light microscope to view the blood smear preparation. The use of a 40x objective is recommended for erythrocyte measurements.

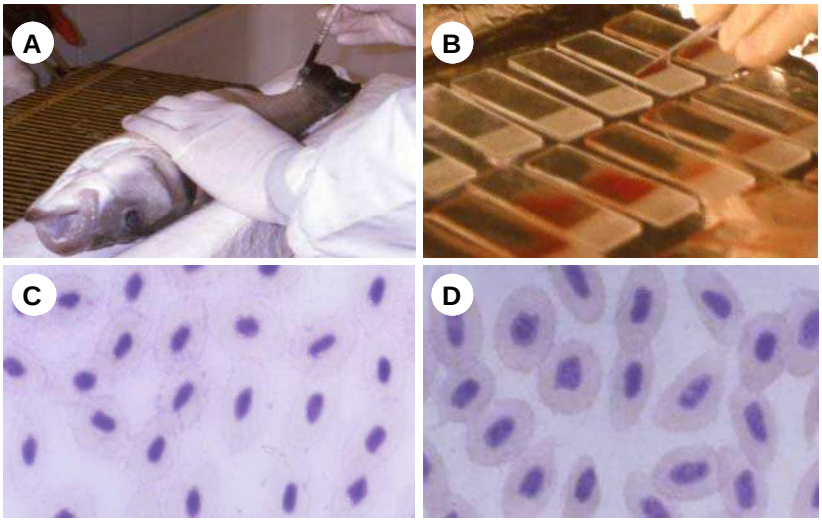


Fig. K.1. Determination of triploid sea bass by measuring erythrocyte sizes. (A) Caudal punction using a 1-ml insulin syringe to obtain a blood sample. (B) Blood extension on a clean glass slide. Staining of erythrocytes with Giemsa solution to see the effect of ploidy on erythrocyte size in diploids (C) and triploids (D).

IV - Materials and equipment

- 1-ml insulin syringe
- Needles (diameter depending on fish size)
- Clean glass slides
- Cover glass
- Gloves and lab coat

V - Reagents and solutions

- Ethanol
- 15% Giemsa solution prepared in phosphate buffer (0.01 M, pH 7.0)
- Distilled water
- Xylen
- DPX

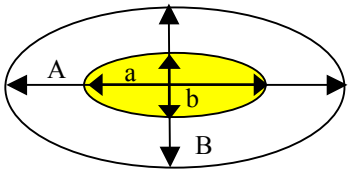
VI - Results and discussion

The effect of triploidy on the erythrocyte cellular and nuclear volume dimensions from the blood smears is highly significant in sea bass (Table K.1). Unlike mammals, erythrocytes in fish species have a nucleus. Erythrocytes and their nucleus are assumed to have an ellipsoidal shape. Accordingly, cellular and nuclear volume of erythrocytes can be estimated by the formula of an ellipsoid using the conventional formula $V = 4/3\pi ab^2$ (Fig. K.2). Comparative

analysis between erythrocyte measurements and karyologically determined ploidy levels confirmed that erythrocyte measurements are an accurate approach to estimate ploidy in the sea bass (Felip *et al.*, 1997).

Table K.1. Erythrocyte measurements from diploid and triploid sea bass (volume, µm^3)

Type of cell	2n	3n	Ratio 2n / 3n	Signif. level
Erythrocytes				
Cell volume	1282.31 ± 20.70	1981.85 ± 33.94	**1.54**	$P < 0.001$
Nuclear volume	144.54 ± 3.35	224.73 ± 5.62	**1.55**	$P < 0.001$



$V_{erythrocyte} = 4 / 3 \times \pi \times (A / 2) \times (B / 2)^2$

$V_{nucleus} = 4 / 3 \times \pi \times (a / 2) \times (b / 2)^2$

where:

A = major axis of erythrocyte

B = minor axis of erythrocyte

a = major axis of nucleus

b = minor axis of nucleus

Fig. K.2. The erythrocyte cellular and nuclear volume in fish can be estimated using the formula of an ellipsoid.

Our data have also supported that the identification of triploid sea bass can be based solely on the measure of the major axis of the cell (Felip *et al*., 1997, 2001a). For these analyses, cell sizes of 35 erythrocytes from 16 fish per ploidy were measured under a light microscope evidencing that this variable was sufficient to detect differences between triploid and diploid fish. Results showed that the major axis of erythrocytes was larger than that of the diploids (13.0 ± 0.49 µm vs 10.2 ± 0.44 µm; $P<0.001$) (Felip *et al*., 1997). Since major axis measurements had a good discriminating power to determine ploidy in the sea bass, that variable could be used alone for estimation of ploidy in practical applications in commercial fish farms. As shown in Fig. K.1, it is a quick and easy procedure that does not require special equipment or expertise in the laboratory.

Furthermore, cell measurements from male germ cells (i.e., spermatogonia and spermatocytes) have evidenced that triploids may also be distinguished from diploids in the sea bass (Felip *et al.*, 2001c), although this particular analysis requires the slaughter of the fish. Experiments in our laboratory using cold-shock treatments for inducing triploidy in sea bass were effective (80%

survival and 90-95% triploidy) and mosaic polyploids were not observed. Of the three techniques employed in our research (karyotyping, NOR analysis and erythrocyte measurements; Protocols *I, J, K*, respectively), sizing of erythrocytes is the most practical for the routine screening of triploid sea bass when the number of fish under experimentation is reduced. However, it should be noted that previously to its application, this procedure needs to be verified by karyotypical analysis.

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