Protocol J - Silver staining of NOR regions [Pratical guide of protocols: methods of verification of the ploidy]

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I - Introduction

The nucleolar organizer regions (NORs) contain the ribosomal genes (i.e., 18S and 28S rRNA). During metaphase they are within the "stalks" (i.e., the secondary constrictions) of chromosomes, whereas during interphase they are located in the fibrillar components of the interphasic nucleolus where the rRNA-protein complex synthesized by active genes prior to interphase is localized. Many reports suggest that these proteins play an important role in its decondensation and/or in its transcription and thus, finally in the maturation of the rRNA.

The proteins associated with NOR regions are known to be argyrophilic. Cytological tests have demonstrated that this strong affinity for silver nitrate only occurs for the decondensed state of NOR chromatin, either transcribing or ready for transcription (Fakan and Hernández-Verdún, 1986). Consequently, protocols for analysis of active NORs in nuclei regularly use silver nitrate solutions to localize NOR sites during active transcription of interphasic nucleoli of animal and plant chromosomes. A protective colloidal developer is also used in this procedure to control the reduction of silver (Howell and Black, 1980).

Since in somatic interphase cells there is a close relationship between the number of visible nucleoli and the number of chromosome sets, nucleoli counting has been used as an indirect method for identification of ploidy levels in fish. Nevertheless, some exceptions occur evidencing that the validity of this procedure needs to be verified in each teleost species to make identification of triploids unambiguous. Accordingly, patterns of chromosomal NOR variation in fishes have been demonstrated in cyprinids (Carman *et al*., 1992), salmonids (Phillips *et al.*, 1989) and flatfish (Piferrer *et al.*, 2000). Analyses on the maximum number of nucleoli at different somatic tissues, ages, sexes and ploidy level in common carp have showed considerable variation. Thus, it has been evidenced that the maximum number of nucleoli per cell detected using silver nitrate increased as the fish became older (Carman *et al*., 1992). However, despite complications to the practical application of this approach in fish, ploidy determination is possible by counting NOR sites in interphase nuclei when the use of NOR analysis is validated to determine ploidy as previously demonstrated in turbot (Piferrer *et al*., 2000).

Finally, the study of Flajshans *et al.* (1992) has reported the minimum number of cells necessary to be analysed to determine ploidy level. A regression analysis has shown that a sample of 80 cells exhibiting three active NORs is sufficient to confirm triploidy in manipulated fish species. The method developed in this Protocol is modified from Kligerman and Bloom (1977) and Rufas *et al*. (1982) in order to count interphasic nucleoli from newly hatched larvae and identify triploid sea bass.

II - Purpose

A method for the preparation of NOR regions is described in sea bass for counting interphasic nucleoli from newly hatched larvae. NORs are visualized by using silver staining to verify the ploidy condition in this species.

III - Procedure

(i) Place the newly hatched larvae (n ~ 12) into a 250-ml beaker containing distilled water solution. Mix well and pour off distilled water adding fresh distilled water. Incubate for 30 minutes shaking samples occasionally during the incubation.

(ii) Discard the distilled water and fix the larvae using a cold mixture of 3:1 methanol:acetic acid for 30 minutes. Repeat twice. Tissues may be stored in fixative for several months at 4ºC.

(iii) Add 50 µl of 50% acetic acid in each well of a 96-well plate. Add one larva per well.

(iv) Take 40 µl of each well and gently pipette up and down until larvae is disaggregated. A multichannel pipette can be used.

(v) Clean micro slides using a 1:1 ether:ethanol mixture and prewarm micro slides on a heat plate at 50-60ºC.

(vi) Take 5 µl of the cell suspension and place on a precleaned and prewarmed micro slide. Repeat this process forming 2-3 rings of cells. Allow preparations to dry 10-15 minutes and store at room temperature until staining.

(vii) Wash the preparations and the material to be used in a formic solution before use. Allow preparations to air dry for 10-15 minutes.

(viii) Prepare a fresh silver nitrate solution just before use: Solution A and B (see Reagents and Solutions). Protect these solutions from light using capped amber-glass bottles.

(ix) Add on each prewarmed slide 2 drops of Solution A and 4 drops of Solution B. Mix carefully.

(x) Cover with a cover glass and incubate the micro slides in a hummed chamber at 60ºC for 10 minutes.

(xi) Remove the cover glass using a distilled water bottle. Wash the micro slides well. Allow preparations to air dry for 10-15 minutes. Use a light microscope to view the NOR preparations. The use of a 20x objective is recommended for NOR localization.

IV - Materials and equipment

- Razor blades for chopping larvae
- Excavated glass micro slides
- Clean glass slides
- Paper towel
- Heat plate
- Glass pasteur pipette
- 96-well plates
- Hummed chamber
- Cover glass
- Capped amber-glass bottles
- Light microscope
- Gloves and lab coat

V - Reagents and solutions

- Fixer solution of 3:1 methanol:acetic acid
- 50% acetic acid prepared in water

- Formic acid prepared with 200 ml of bidistilled water and a drop of concentrated formic acid of high purity

- Silver nitrate prepared just before use as follows, and protected from light:

(i) *Solution A* (a colloidal developer solution): dissolve 2 g of gelatin in 100 ml of distilled water and 1 ml of formic acid.

(ii) *Solution B* (an aqueous silver nitrate solution): dissolve silver nitrate in distilled water in a proportion 1:2 (w/v). Store in capped amber-glass bottles or by other light-protective means.

Silver-nitrate solution should be handled with care. The operator should wear gloves during the procedure and protect the work area with paper towels since silver nitrate is a hazardous chemical that may cause burns.

VI - Results and discussion

The counting of nucleolar organizer regions (NORs) is an indirect method used to verify the ploidy level, and consequently for the identification of polyploids. This procedure was applicable to sea bass since this species has been shown to have only one chromosome with a NOR site per haploid set of chromosomes. It is located at the terminal site on the short arm of the acrocentric chromosome pair 22 (Aref´yev, 1989). Results from sea bass showed that haploid individuals had one nucleolus/cell, diploid individuals had one or two nucleoli/cell and triploid individuals had 1, 2 or 3 nucleoli/cell. Accordingly, this procedure was effective for the determination of triploid (Felip *et al*., 1997), haploids and gynogenetic fish in the sea bass (Felip *et al.*, 1999). As shown in Fig. J.1, diploid sea bass exhibit 1 or 2 NORs (Fig. J.1 A) and triploid fish up to 3 NORs (Fig. J.1 B).

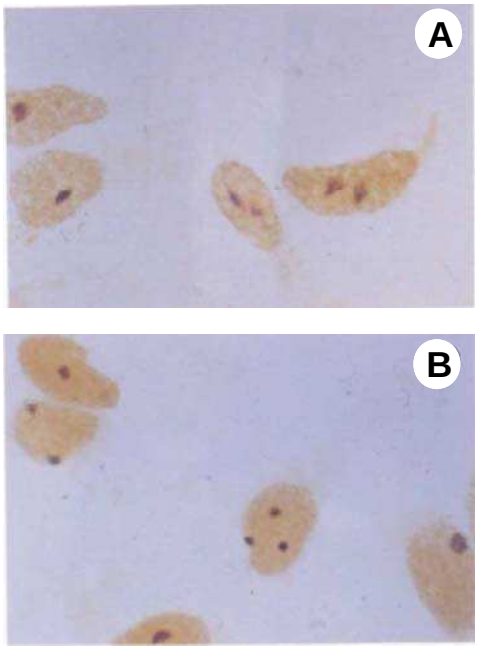

Fig. J.1. Visualization of the Ag-stained nucleolar organizing regions (NORs) in diploid (A) and triploid (B) sea bass.

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