

*Development of primary cell cultures from *Ciona intestinalis* explants*

1. Adult specimens of *Ciona intestinalis*, 3-4 cm large, are sacrificed and neural complexes (including neural ganglion, neural gland and surrounding muscle) are collected using sterile instruments in artificial sea water (ASW) supplemented with 5x penicillin / streptomycin / amphoterycin (PSA).
2. Explants are cleaned from surrounding tissues using sterile instruments, then washed for 1 min in bleach diluted 1:20 in ASW and rinsed 3 x 1 min in ASW supplemented with 5x PSA.
3. Explants are mechanically triturated using sterile micro-scissors then digested for 3-4 h at 4°C under agitation with collagenase P in ASW supplemented with DNasel.
4. Digestion solution is diluted 10 times in ASW and explant fragments are collected by centrifugation (3,000 rpm for 2 min).
5. Supernatant is discarded and fragments are resuspended in a small volume of cell culture medium supplemented with carbohydrates, antibiotics, fetal bovine serum (FBS) and collagenase P then placed in a 12-well plate and incubated at 17°C in a standard incubator.
6. One day later, complete cell culture medium supplemented with collagenase P is added up to 1 mL and explants cultures are further incubated at 17°C.
7. One day later, explants cultures are rinsed once with ASW then incubated in fresh complete cell culture medium containing bovine fibroblast growth factor (bFGF).
8. Culture medium is renewed twice a week using, when available, filtered conditioned medium to complement fresh medium.
9. Adherent cells from 50% confluent cultures are trypsinized at room temperature (using trypsin in calcium-magnesium-free ASW), then harvested, centrifuged and seeded at high density into a 24-well plate containing fresh complete cell culture medium mixed 1:2 with conditioned medium.

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Ciona intestinalis: adult specimens
collected in the Bay of Naples.

Cell culture apparatus: cooled incubator
and biological safety cabinet class II.

Cell culture medium: Leibovitz's medium
(L15), adjusted to sea water salt
concentration

Medium supplements: Fetal bovine serum
(FBS), 0.3 mg/ml L-glutamine, 100 U/ml
penicillin, 0.1 mg/ml streptomycin,
0.25 mg/ml amphoterycin, carbohydrates
(fructose 1.5 mg/ml, galactose
4.3 mg/ml, mannose 1.5 mg/ml, ascorbic
acid 10 mg/ml, taurine 0.12 mg/ml),
0.5 mg/ml collagenase P, 2.5 ng/ml
bovine fibroblast growth factor.

Solutions: artificial sea water (ASW: 460
mM NaCl, 10 mM KCl, 55 mM MgCl₂, 11
mM CaCl₂, 10 mM glucose, 10 mM
HEPES), calcium-magnesium-free
artificial sea water (CMF-ASW: ASW
without 55 mM MgCl₂, 11 mM CaCl₂),
digestion solution (1 mg/ml collagenase
P, 0.1% DNasel in ASW), trypsin solution
(2.5 mg/ml trypsin in CMF-ASW).

Plasticware: cell culture dishes and
serologic pipettes from Corning.

All chemicals were purchased from
Sigma-Aldrich.