Keast Group Protocols

Immunohistochemistry on sections of organs and pelvic ganglia

In this method, sections are mounted directly on slides for subsequent immunohistochemical staining. All data in GUDMAP has been replicated in at least three embryos/animals and representative images uploaded.

Preparation of tissue for cryosectioning

- Fix tissue in 4% paraformaldehyde (PFA)/0.1M PBS (pH 7.2) overnight at 4°C.
- Wash tissue 3X in 0.1M PBS (pH 7.2) for 10 minutes at room temperature.
- If tissue is to be stored prior to cryosectioning, store in 0.1M PBS (pH 7.2) with 0.1% sodium azide at 4°C.
- Incubate tissues 18-24 h in 30% sucrose/0.1M PBS (pH 7.2) at 4°C.
- Embed tissue in OCT and freeze. Cut sections at 14 µm, mounting onto 1% gelatinised slides.
- Air-dry slides for at least 10 minutes.

Immunohistochemistry

Pre-treatment of sections

- Wash slides 2X in 0.1M PBS (pH 7.2) for 10 minutes at room temperature, shaking gently.
- Dry back of slide with Kimwipe and carefully wipe around sections to remove as much PBS as possible from the sections, but not allowing the sections to dry out.
- Apply 100 µl of blocking solution to each slide and incubate at room temperature in a humid chamber for 1-2 hours.
- Blocking solution: 10% non-immune horse serum (Sigma Cat #12449C) + 0.1% Triton in 0.1M PBS (pH 7.2). This solution should be made up on the day, not stored.

Primary Antibody Application

- Dilute the primary antibody in hypertonic PBS (see below). The optimum dilution of the primary antibody should be determined beforehand in a separate study on tissue of the same species prepared in a similar way.
- Wipe off excess blocking solution from around sections and add ~100 µl of primary antibody to each slide, ensuring sections do not dry out. Incubate 18-24 h at room temperature in a humid chamber.
- Wash slides in 0.1M PBS (pH 7.2) for 15 minutes at room temperature, shaking gently.

Secondary Antibody Application

- Dilute the secondary antibody in hypertonic PBS (see below). The optimum dilution of the secondary antibody should be determined beforehand in a separate study on tissue prepared in a similar way.

- Wipe off excess blocking solution from around sections and add ~100 µl of secondary antibody to each slide, ensuring sections do not dry out. Incubate for 2-3 hours at room temperature **in the dark.**
- The optimum dilution of the secondary antibody used is determined for each experiment.

Mounting

- Wash slides in 0.1M PBS (pH7.2) for 15 minutes at room temperature, shaking gently.
- Coverslip slides in commercial anti-fade mountant (e.g., Vectashield). Seal edges of coverslip with nail polish.

Solutions

Solutions A and B (below) are used for making two types of PBS:

Solution A: (0.2M): 24.0g NaH_2PO_4 / 1000 ml H_2O

Solution B: (0.2M): 28.4g Na_2HPO_4 / 1000 ml H_2O

Phosphate Buffered Saline (PBS) 0.1M (pH 7.2)

1) 140 ml Sol A + 360 ml Sol B + ~450 ml H_2O + 8.5g NaCl. Dissolve.
2) pH to 7.2 with 1M NaOH/HCl
3) Make up to 1 litre with H_2O

Hypertonic PBS (pH 7.2)

1) 70 ml Sol A + 180 ml Sol B + ~200 ml H_2O + 8.5g NaCl and dissolve
2) pH to 7.2 with 1M NaOH/HCl
3) Make up to 500 ml with H_2O

Antibody Lists

Primary antibody list including supplier details

Gene Symbol (MGI)	Antibody Name	Supplier / Catalogue Number
Calca	Calcitonin gene-related peptide (CGRP) antibody; host rabbit	Sigma / C8198
Calca	Calcitonin gene-related peptide (CGRP) antibody; host rabbit	Millipore (Chemicon) / AB5920
GFP	Green fluorescent protein (GFP) antibody; host goat	Rockland Antibodies & Assays / 600-101-215
Elavl3	HuC antibody; host Human	Gift. No longer available.
Nefh	Neurofilament-200 antibody; host rabbit	Sigma / N4142
Nos1	Neuronal nitric oxide synthase (nNOS) antibody; host Rabbit	Invitrogen / 61-7000
Tac1	Substance P antibody; host rabbit	ImmunoStar / 20064
Th	Tyrosine hydroxylase (TH) antibody; host sheep	Chemicon (Millipore) / AB1542
Tubb3	Neuronal Class III β-Tubulin (TUJ1) antibody; Rabbit Monoclonal	Covance / MRB-435P
Uchl1	Human protein gene product 9.5 (PGP9.5) antibody; host rabbit.	Cederlane Laboratories Limited / CL95101
Vip	Vasoactive intestinal peptide (VIP) antibody; host rabbit	DiaSorin / 20077
Acta2	α-smooth muscle actin (SMA) antibody; host mouse	Sigma / A5228

Secondary antibody list including supplier details

Secondary Antibody	Tag	Supplier / Catalogue Number
Donkey Anti-Goat IgG (H+L) antibody	Alexa Fluor® 488	Invitrogen / A-11055
Donkey Anti-Rabbit IgG (H+L) antibody	Alexa Fluor® 488	Invitrogen / A-21206
Donkey Anti-Sheep IgG (H+L) antibody	Alexa Fluor® 488	Invitrogen / A-11015
Donkey Anti-Goat IgG (H+L) antibody	Alexa Fluor® 594	Invitrogen / A-11058
Donkey Anti-Rabbit IgG (H+L) antibody	Alexa Fluor® 594	Invitrogen / A-21207
Donkey Anti-Human IgG (H+L) antibody	Alexa Fluor® 594	Jackson ImmunoResearch Laboratories Inc / 709-585-149
Donkey Anti-Rabbit IgG (H+L) antibody	Fluorescein isothiocyanate (FITC)	Jackson ImmunoResearch Laboratories Inc / 711-001-003