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Hypoxyprobe™-1 Omni TIMES Kit

(HPI Catalog # HP17-XXX)

Kit contents:

Solid pimonidazole HCl (Hypoxyprobe™-1)and affinity purified rabbit antipimonidazole antibody (PAb2627AP)(ca 0.5 mg/mL IgG), as well as Anti Rabbit HRP secondary antibody with the required activating DAB HRP chromogen.

Applications: Immunochemical detection of cell and tissue hypoxia including immunofluorescence, immunoperoxidase; Western blotting; or, flow cytometry. Very low background in mouse tissues when the Hypoxyprobe™-1 Omni Kit is combined with an the Anti Rabbit HRP secondary antibody with DAB HRP chromogen.

Quantities:
a. Hypoxyprobe™-1 Omni Kits contain100 mg, 200 mg or 1000 mg of Hypoxyprobe™1 (pimonidazole HCl). Typically a dosage of 60mg/kg body weight is used for small animal studies.
b. One vial (100 and 200 mg Kits) or two vials (1000 mg Kit) of 200 microliters of affinity purified anti-pimonidazole rabbit antisera containing 0.09% sodium azide and 1% BSA as stabilizers. Optimal dilution of PAb2627AP is to be determined by each investigator but a 1/50 -1/200 dilution has been found to give strong immunostaining in mouse tumor tissue when combined with the peroxidase conjugated goat anti-rabbit hrp secondary antisera.

Storage:
a. Store Hypoxyprobe™-1 solid at room temperature in the dark (can also be stored at 2-8 degrees C).
b. Store rabbit PAb2627AP, Anti Rabbit hrp and DAB HRP chromogen at 2-8 degrees C. **Do not freeze**

Details of Hypoxyprobe™-1 Omni TIMES Kit components

1) Hypoxyprobe™-1 is a substituted 2-nitroimidazole whose chemical name and only ingredient is pimonidazole hydrochloride. Hypoxyprobe™-1 has a molecular weight of 290.8; a water solubility of 400 millimolar equivalent to 116 mg/ml; and, ultraviolet absorbance at 324 nm with an extinction coefficient of 7020 in 0.9% saline. The free base, pimonidazole, has a molecular weight of 254.3, a pKa of 8.7 and an octanol water partition coefficient of 8.5. See www.hypoxyprobe.com for mechanism of action, frequently asked questions (FAQ) and applications for Hypoxyprobe™-1 kits.

Hypoxyprobe™-1 is chemically stable in both solid form and aqueous solution. For example, solid Hypoxyprobe-1 has been stored for two years at room temperature in subdued light without detectable degradation as assessed by UV and HPLC analyses. Hypoxyprobe™-1 solutions in 0.9% saline have been stored at a concentration of 100 gms/liter at 4°C for 4.5 years without detectable degradation (UV and HPLC analyses).

Pimonidazole is reductively activated in hypoxic cells. The activated intermediate forms stable covalent

adducts with thiol (sulphydryl) groups in proteins, peptides and amino acids. The antibody reagent PAb2627AP binds to these adducts allowing their detection by immunochemical means. See www.hypoxyprobe.com for mechanism of action, frequently asked questions (FAQ) and applications for Hypoxyprobe™-1 kits.

2) PAb2627AP is an affinity purified rabbit antibody preparation that binds to protein adducts of Hypoxyprobe™-1 in hypoxic cells. PAb2627AP contains sodium azide for added stability. Tissues of interest can be studied by immunohistochemistry of frozen fixed or formalin fixed paraffin embedded sections; Western blotting; or, flow cytometry following tissue disaggregation.

Note: PAb2627AP binds to protein, peptide and amino acid adducts of pimonidazole in hypoxic cells but tissue processing for immunohistochemical assay washes out small molecule peptide and amino acid adducts so that immunohistochemical hypoxia detection relies on protein adducts of pimonidazole in hypoxic tissue.

Assay Instructions

1. Investigations of normal or tumor tissue hypoxia begin with the intravenous infusion, intraperitoneal injection or oral ingestion of a Hypoxyprobe™-1 solution at a dosage of 60 mg/kg body weight. For a 25 gram mouse this amounts to 1.5 mg/mouse. Dosages up to 400 mg/kg have been used in mice without detectable toxicity or change in tissue hypoxia but 60 mg/kg gives good immunostaining at minimum cost. The solubility of Hypoxyprobe™-1 in saline is 116 mg/ml so that very small volumes can be used to administer Hypoxyprobe™-1.

Following injection or ingestion, Hypoxyprobe™-1 distributes to all tissues including brain but it forms adducts with thiol containing proteins only in those cells that have a oxygen concentration less than 14 micromolar -- equivalent to a partial pressure pO2 = 10 mm Hg at 37°C. In addition to tumors, normal tissues such as liver, kidney and skin possess cells at, or below, a pO2 of 10 mmHg. These normal tissues bind Hypoxyprobe™-1.

The plasma half-life of Hypoxyprobe™-1 in mice is approximately 25 minutes (see the FAQ link at www.hypoxprobe.com for references). For comparison, plasma half-lives for rats is 45 minutes, dogs 90 minutes and humans 300 minutes. Mouse tissues of interest may be harvested 15 to 90 minutes after Hypoxyprobe™-1 administration. Hypoxprobe™-1 residing in tissues at the time of harvest will be bound when dissected tissues go anoxic. However, the amount of residual Hypoxyprobe™-1 is very small compared to the amount that tissues are exposed to during a 15 to 90 minute experiment so that any non-specific binding due to residual Hypoxyprobe™-1 is undetectable.

In addition to animal studies, Hypoxyprobe™ kits can be used for cells in tissue culture (see Applications link at www.hypoxprobe.com). Typically, cell suspensions are incubated under hypoxia for 1 to 2 hours in the presence of 100 to 200 micromolar Hypoxyprobe™-1. The cells are harvested by cytospin, fixed and immune-stained with the supplied anti rabbit hrp and chromogen or a fluorescent secondary reagent.

Suggested protocol for PAb2627 immunohistochemistry

Kleiter et al., Int. J. Radiat. Oncol. Biol. Phys. 64: 592-602, 2006.

Hypoxyprobe™ technology is robust and investigator-initiated modifications are encouraged.

Step	Procedure	Time, min.	Temp.	Reagents	Notes
1	Warm paraffin tissue section	20	40 °C	None	
2	Dip and blot 10 times	2	RT	Clear-Rite 3	1
3	"	2	RT	100% Ethanol	
4	"	2	RT	95% Aqueous ethanol	
5	"	2	RT	80% Aqueous ethanol	
6	"	2	RT	0.2% Brij 35 in distilled water	2
7	"	2	RT	PBS+ 0.2% Brij 35	3
8	Quench tissue peroxidase	5	RT	3% H2O2 in distilled water	4
9	Wash	2	RT	PBS+ 0.2% Brij 35	
10	Antigen retrieval	20	90 °C	Target retrieval reagent	5,6
11	Cool to RT	20	RT	None	
12	Wash	2	RT	PBS + 0.2% Brij 35	7

13	Block non-specific binding	5	RT	Protein blocking agent	8,9
14	Rabbit PAb2627AP 1^{o} reagent	60	RT	PAb2627AP(1:50-1:200)	10
15	Wash	2	0 ^{o}C	PBS + 0.2% Brij 35	7
16	Anti-rabbit 2 ^{o} reagent	20	RT	Anti-rabbit polymer HRP	11
17	Wash	2	0 ^{o}C	PBS + 0.2% Brij 35	7
18	Peroxidase chromogen	10	RT	DAB	12
19	Wash	2	RT	Running distilled water	
20	Counterstaining	0.5	RT	Hematoxylin	13
21	Wash	2	RT	Running distilled water	
22	Cover tissue sections	45	45 ^{o}C	Aqueous CC/Mount	14

Technical Notes

1. Clear-Rite 3 -- a less toxic alternative to xylene -- is available from VWR (Cat# 84000-052). For dewaxing (step 2) and tissue rehydration (steps 3-7), ProbeOn Plus slides (Fisher Scientific; Cat# 15-188-52) are held vertically in a MicroProbe Staining Station (Fisher Scientific) in pairs with tissue sections facing each other. The paired slides are dipped in solvent allowing capillary action to carry solvents over the tissue sections. Solvent is removed by blotting the lower end of the slide pair on adsorbent filter paper. The dip and blot procedure is repeated a total of 10 times for each of steps 2-7.

Note: These steps were designed for the MicroProbe Staining Station but other routine IHC procedures can be used.

2. Brij 35 is enzyme grade polyoxyethylene(23)lauryl ether available from Fisher Scientific (Cat# BP345-500). Alternatives to Brij35 such as Tris buffered saline (TBS) available from Chemicon International, Temecula, CA (Cat# 20845) can be used as the rinse solution. It is recommended that Tween 20® be added to the TBS rinse buffer at a final concentration of 0.1%.
3. PBS is 10 mM phosphate buffered saline prepared from tablets available from Sigma (Cat# P-4417).
4. 3% H2O2 is diluted Analytical Reagent grade 31.3% H2O2 available from Malinckrodt Baker, Paris, KT (Cat# 5240). Commercial peroxidase blockers may be used.
5. Antigen retrieval agents such as AbD Serotec Cat# BUF025B; Chemicon International Cat# 21545; or, DAKO Cat# S2369 can be used. . Pimonidazole protein adducts are very robust so that the antigen retrieval reagent can be chosen on the basis of requirements for other factors of interest in tissue sections.
6. Slides held vertically in slide incubator.
7. Slides washed with magnetically stirred PBS + 0.2% Brij 35 in a rectangular staining jar.
8. For example, serum free protein blocker from DAKO Corp. (Carpinteria, CA)(Cat# X0909).
9. Slides held horizontally for steps 13-20 so as to limit non-specific, edge staining of the sections.
10. PAb2627AP diluted 1:50-1:200 in 10 mM PBS containing 0.2% Brij 35 or other suitable antibody diluent such as Chemicon International, Cat# 21544. Typically, 100 uL of diluted PAb2627AP solution is applied to each tissue section.
11. For example, DAKO EnVision+System kit K4011.
12. For example, DAKO EnVision+System kit K4011.
13. Any commercially available hematoxylin counterstain is suitable including Chemicon International Cat# 20844.
14. CC/Mount (Sigma; Cat# C9368), a direct replacement for Biomeda's Crystal Mount, is an aqueous based permanent mount for immunostained sections. Alternatives include cover slipping with Permount (Fisher Scientific; Cat# SP15-500).

Procedure for Frozen Tissue Sections:

Most of the published work reporting fluorescence immunohistochemical detection of pimonidazole adducts is based on frozen sections and much of the data comes from Dr. A. J. van der Kogel's laboratory in Nijmegen. The tumor or tissue specimen is collected and directly frozen in liquid nitrogen until cryosectioned into 4 um sections. Consecutive sections are cut at the largest circumference of the tissue. The sections are then stored at -80^{o}C until stained. After thawing, the sections are fixed in cold acetone (4^{o}C) for 10 min. The sections are rinsed and incubated overnight at 4^{o}C with mouse monoclonal anti-pimonidazole antibody (clone 4.3.11.3)(MAb1) diluted in PBS containing 0.1% bovine serum albumin c and 0.1% Tween 20 – the extent of dilution determined by investigator. The sections are then incubated for 90 min with Cy-3-conjugated goat anti-mouse antibody 1:150 (Jackson Immuno Research Laboratories). Between all steps of the staining procedure, the sections are rinsed three times with for 2 minutes in PBS.