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Hypoxyprobe™ F6 Kit

HPI Catalog # HP4-XXX

Kit Contents:

Solid CCI-103F (Hypoxyprobe™-F6)
Diluted Rabbit anti-CCI-103F antisera (PAbF6)

Applications: Use the Hypoxyprobe™-F6 Kit to measure hypoxia in tissues and cells by means of immunochemical detection including immunoperoxidase, immunofluorescence or flow cytometry. (Kleiter et al., Int. J. Radiation Oncol. Biol. Phys. 64(2): 592-602, 2006). Ljungkvist et al., Int. J. Radiat. Oncol. Biol. Phys. 62(4): 1157-1168, 2005).

Quantities: a. Hypoxyprobe™-F6 Kits contain 100, 200 or 1000 mg, respectively, of CCI-103F (Hypoxyprobe™–F6). Typical doses are 60mg/kg body weight is used in small animal studies.

b. Each Kit contains diluted anti-CCI-103F rabbit antiserum (PAbF6) containing 0.09% sodium azide and 1% BSA as stabilizers. The 100 and 200 mg Kits contain one 200 uL unit of antiserum; the 1000 mg Kit contains two 200 uL units of antiserum. Optimal operating dilutions of the antisera are to be determined by investigators but 1/200 -1/500 dilutions give strong immunoperoxidase staining when combined with peroxidase conjugated goat anti-rabbit secondary antisera.

Not supplied: Anti-rabbit secondary reagents or other immunohistochemical reagents (buffers, antigen retrieval agents, etc).

Storage: a. Store Hypoxyprobe™–F6 in the dark at room temperature (can also be stored at 2-8 degrees C)
b. Store rabbit antisera (PAbF6) at 2-8 degrees C.

Detailed description of Hypoxyprobe™-F6 Kit components

1) Hypoxyprobe™-F6 (CCI-103F) is a hexafluorinated 2-nitroimidazole with six magnetically equivalent fluorine atoms; a molecular weight of 337.2; and, a water solubility of 5.3 millimolar equivalent to 1.8 mg/ml. CCI-103F is typically administered intraperitoneally in a 10% DMSO/ peanut oil mixture (Ljungkvist et al., Int. J. Radiat. Oncol. Biol. Phys. 62(4): 1157-1168, 2005). CCI-103F is very stable when stored in subdued light.

CCI-103F is reductively activated in hypoxic cells. Activated intermediates forms stable covalent adducts with thiol (sulphydryl) groups in proteins, peptides and amino acids. Primary anti-CCI-103F antisera bind to these adducts allowing their detection by immunochemical means. See www.hypoxyprobe.com for mechanism of action and frequently asked questions (FAQ) about the use of Hypoxyprobe™ markers in general.

2) Anti-CCI-103F rabbit antiserum (PAbF6) binds to protein adducts of reductively-activated CCI-103F and is supplied as diluted rabbit antiserum containing 0.09% sodium azide and 1% BSA as stabilizers. Tissues of interest can be studied by immunohistochemistry on formalin fixed paraffin embedded sections or frozen fixed sections, by flow cytometry following tissue disaggregation, or by Western blotting. The anti-CCI-103F antisera binds to protein, peptide and amino acid adducts of CCI-103F but tissue processing in preparation for immunohistochemical assays washes out peptide and amino acid adducts so that immunohistochemical detection relies only on the protein adducts of CCI-103F in hypoxic tissue.

Immunoperoxidase analysis on formalin-fixed, paraffin-embedded tissue sections involves incubating tissue sections with 100 microliters of a ca 1:500 dilution of anti-CCI-103F antisera. A chromogenic secondary goat anti-rabbit secondary is then applied to reveal the distribution of the hypoxia marker adducts. A detailed immunoperoxidase protocol is provided below as a general guide ***but it is emphasized that Hypoxyprobe™ technology is robust and investigator-initiated modifications are encouraged.*** Please see Ljungkvist et al., Int. J. Radiat. Oncol. Biol. Phys. 62(4): 1157-1168, 2005 and references therein for studies on frozen sections.

Assay Instructions

Marker investigations in rodents begin with an iv intraperitoneal injection of CCI-103F dissolved in 10% DMSO/90% peanut oil (see Ljungkvist et al., Int. J. Radiat. Oncol. Biol. Phys. 62(4): 1157-1168, 2005) at a dosage of 60 mg/kg. Sixty to 120 minutes later, the tissue of interest is harvested. A dosage of 60 mg/kg for CCI-103F is less on a molar basis than that for pimonidazole HCl, the other hypoxia marker supplied by Hypoxyprobe, Inc but this is compensated by a longer plasma half-life for CCI-103F ($t_{1/2}$ = 90 minutes versus 25 minutes for pimonidazole in mice).

Following injection, Hypoxyprobe™ markers distribute to all tissues but form adducts with thiol containing proteins, peptides and amino acids only in those cells that have a oxygen concentration less than 14 micromolar -- equivalent to a partial pressure pO_2 = 10 mm Hg at 37ºC. Normal tissues such as liver, kidney and skin possess cells at, or below, a pO_2 of 10 mmHg and these tissues will bind Hypoxyprobe™ markers. Unbound Hypoxprobe™ markers residing in tissues at the time of harvest will be activated and bind when dissected tissues go anoxic. However, residual Hypoxyprobe™ marker concentrations are very small compared to the amount that tissues are exposed to during a typical experiment so that any non-specific binding from residual Hypoxyprobe™ marker at the time of tissue harvest is undetectable. In addition to animal studies, Hypoxyprobe™ kits can be used for cells in tissue culture (see Applications link at www.hypoxyprobe.com).

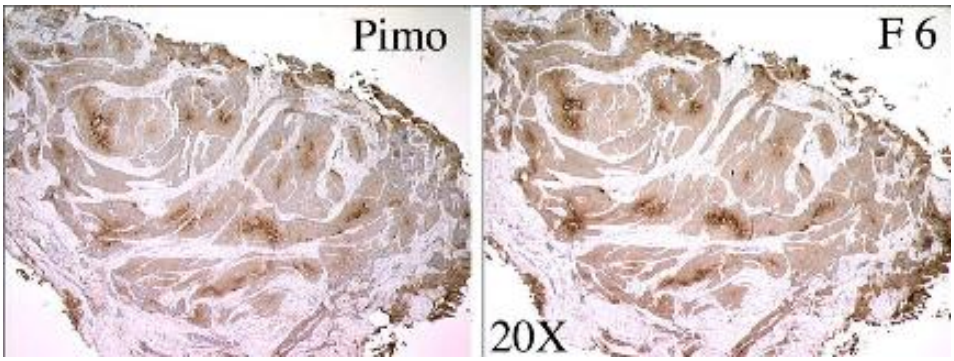

Figure. Comparison of CCI-103F binding (F6, right panel) with pimonidazole binding (Pimo, left panel) in adjacent tissue sections from a formalin fixed, paraffin embedded canine tumor biopsy. The immunoperoxidase data show that F6 binding is very similar to that for pimonidazole. The rabbit antisera for F6 and pimonidazole adducts are non-cross reacting. This allows independent measurements of the binding of the two markers (see, Kleiter et al., Int. J. Radiation Oncol. Biol. Phys. 64(2): 592-602, 2006).

Suggested protocol for immunoperoxidase analysis of CCI-103F binding in formalin fixed paraffin embedded tissue.

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	40ºC	None	
2	Dewax, Dip and Blot x 10	2	RT	Clear-Rite 3	1
3	Rehydrate, Dip and Blot x 10	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% H_2O_2 in distilled water	4
9	Wash	2	RT	PBS+ 0.2% Brij 35	
10	Antigen retrieval	20	90º	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	F6 primary antisera	60	RT	Primary antisera (1:200-1:500)	10
15	Wash	2	0º	PBS + 0.2% Brij 35	7
16	Anti-rabbit 2^{o} reagent	20	RT	DAKO anti-rabbit polymer HRP	11
17	Wash	2	0º	PBS + 0.2% Brij 35	7
18	Peroxidase chromogen	10	RT	DAB peroxidase substrate	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	45ºC	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% H_2O_2 is diluted Analytical Reagent grade 31.3% H_2O_2 available from Malinckrodt Baker, Paris, KT (Cat# 5240). Commercial peroxidase blockers may be used.

5. Antigen retrieval agents such as AbD Serotech 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. Rabbit antisera diluted 1:50-1:200 in 10 mM PBS containing 0.2% Brij 35 or other suitable antibody diluant such as Chemicon International, Cat# 21544. Typically, 100 uL of diluted rabbit antisera 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 reagent 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 immunostaining CCI-103F adducts in frozen, fixed tissues.

Extensive fluorescence immunohistochemical studies of Hypoxyprobe™-F6 binding have been published by Dr. A. J. van der Kogel et al from their laboratory in Nijmegen. For guidance please see Ljungkvist et al, Int. J. Radiat. Oncol. Biol. Phys., 62(4): 1157-1168, 2005 and references therein.