Materials and Reagents:

I. Deparaffinization / Rehydration

1. Tissue sections
2. Xylene
3. 100% ethanol
4. 95% ethanol

II. Antigen Unmasking

A. Domestic (850W) or scientific microwave

B. Microwaveable vessel with slide rack to hold approximately 400-500 ml

C. Epitope antigen retrieval buffer:

1. Heat-induced retrieval solution (HIER)

(1) Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0):

- Tri-sodium citrate (dihydrate) --------- 2.94 g
- Distilled water ---------- 1000 ml

Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4 C for longer storage.

(2) EDTA Buffer (1mM EDTA, 0.05% Tween 20, pH 8.0):

- EDTA ---------- 0.37 g
- Distilled water ---------- 1000 ml

Mix to dissolve. Adjust pH to 8.0 using 1N NaOH. Then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4 C for longer storage.

(3) Tris- EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0):

- Tris Base ---------- 1.21 g
- EDTA ---------- 0.37 g
- Distilled water ---------- 1000 ml (100 ml to make 10x, 50 ml to make 20x)

Mix to dissolve. pH is usually at 9.0 and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4 C for longer storage.

2. Proteolytic enzyme digestion reagent (PIER)

(1) Pepsin Solution

Pepsin Stock Solution (1% in 10mM HCl):

- Pepsin ---------- 100 mg
- 10mM HCl (pH 2.0) ---------- 10 ml

Mix to dissolve. Store at -20 ºC

Pepsin Working Solution (0.5% in 5mM HCl):

- Pepsin Stock Solution (0.5%) ---------- 1 ml
- Distilled water ---------- 1 ml

Mix well.

(2) Proteinase K Solution (20 ug/ml in TE Buffer, pH 8.0, for membrane antigens such as integrins, CD31, vWF, etc):

TE Buffer (50mM Tris Base, 1mM EDTA, 0.5% Triton X-100, pH 8.0):

- Tris Base ---------- 6.10 g
- EDTA ---------- 0.37 g
- Triton X-100 ---------- 5 ml
- Distilled water ---------- 1000 ml

Mix to dissolve. Adjust pH 8.0 using concentrated HCl (10N HCl). Store at room temperature.

Proteinase K Stock Solution (20x, 400 ug/ml or 12 units/ml):

- Proteinase K (30 units/mg)---------- 0.008 g (8 mg)
- TE Buffer, pH8.0 ---------- 10 ml
- Glycerol ---------- 10 ml

Add proteinase K to TE buffer until dissolved. Then add glycerol and mix well. Aliquot and store at –20 ºC for 2-3 years.

Working Solution (1x, 20 ug/ml or 0.6 units/ml):

- Proteinase K Stock Solution (20x) ----- 1 ml
- TE Buffer, pH8.0 ---------- 19 ml

Mix well. This solution is stable for 6 month at 4 ºC.

(3) Trypsin Solution

Trypsin Stock Solution (0.5% in Distilled Water):

- Trypsin ---------- 50 mg
- Distilled water ---------- 10 ml

Mix to dissolve. Store at -20 ºC.

Calcium Chloride Stock Solution (1%):

- Calcium chloride ----------0.1 g
- Distilled water ---------- 10 ml

Mix well and store at 4 ºC.

Trypsin Working Solution (0.05%):

- Trypsin stock solution (0.5%) ---------- 1 ml
- Calcium chloride stock solution 1% ----- 1 ml
- Distilled Water ---------- 8 ml

Adjust pH to 7.8 with 1N N NaOH. Store at 4 ºC for one month or -20 ºC for long term storage

The use of enzyme digestion method may destroy some epitopes and tissue morphology

III. Blocking

A. Peroxidase Blocking Soluiton (3% H_2O_2 in PBS):

- 30% H_2O_2 ---------- 10 ml
- 1XPBS ----------90 ml

Mix well and store at 4 ºC.

B. Universal Blocking Buffer:

- 1%BSA (blocking & stabilizer)
- 0.5% Triton X-100 (penetration enhancer)
- 0.05% sodium azide (preservative)
- 0.01M PBS, pH 7.2-7.4

Mix well and store at 4 ºC.

IV. Staining

A. Primary antibody
B. Conjugated secondary antibody
C. ABC reagents
D. Hematoxylin

Procedures:

Deparaffinization / Rehydration:

1. Wash slides 3X in xylene for 5 min at room temperature.
2. Wash slides 2X in 100% alcohol for 3 min at room temperature.
3. Wash slides 1X in 95% alcohol for 3 min at room temperature.
4. Wash slides 1X in 70% alcohol for 3 min at room temperature.
5. Wash slides 1X in 50% alcohol for 3 min at room temperature.
6. Gently rinse slides using distilled H2O for 5 min at room temperature.

Antigen retrieval:

** Chose one method to perform, or combination of heat mediated and proteolytic enzyme method is an alternative approach when performing double or triple labeling of two or more antigens simultaneously.*

A. Heat-induced epitope retrieval (HIER)

1. Add the appropriate Heat-induced retrieval solution (HIER; Ex, Sodium Citrate Buffer pH 6.0) to the microwaveable vessel, and pre-heat 5 min.
2. Put the slides to the microwaveable vessel in pre-heat antigen retrieval solution for 20 min.
3. Cool slides with buffer on bench top for 20 min
4. Wash slides in PBS for 5 min x 2 at room temperature.

B. Proteolytic enzyme digestion reagent (PIER)

1. Digest samples by Proteolytic enzyme digestion reagent (PIER; EX, Pepsin Solution) for 10 min at 37°C.
2. Wash slides in PBS for 5 min x 2

Blocking:

1. Quench endogenous peroxidase by 3% H_2O_2 in PBS for 10 min at RT

   (If using a HRP conjugate for detection, this step could be performed here or alternatively after primary

antibody incubation.)

2. Wash the slides 3 times with distilled water, 2 minutes each.
3. Block each section with Universal Blocking Buffer for 30 min at RT.
4. Wash slides in PBS for 5 min x 2

Immunostaining:

1. Remove blocking buffer and add primary antibody at proper dilution with 1% BSA containing TBS/PBS and incubate for one hour at RT or overnight at 4°C.
2. Remove antibody solution and wash slides in PBS for 5 min x 3.
3. Quench endogenous peroxidase by 3% H_2O_2 in PBS for 10 min at RT
   (If using a HRP conjugate for detection, this step could be performed here or alternatively before Universal Blocking Buffer blocking.)
4. Secondary antibody should diluted to the concentration based on the manufacturer datasheet in TBS with 1% BSA. Add diluted enzyme-conjugated secondary antibody, and incubate for 30 min to 1h at RT.
   (Prepare ABC reagent according to the manufacturer's instructions and mix solution for 30 min at RT).
5. Remove secondary antibody solution and wash slides in PBS for 5 min x 2.
6. Add ABC reagent to each slide and incubate for 30 min at RT.
7. Remove ABC reagent and wash slidess in PBS for 5 min x 2.
8. *Add DAB to each slide and monitor staining closely.*
9. *As soon as the sections developed*, immerse slides in ddH_2O, or rinse in running tap water for 5 min.
10. Stain slides 10-30 seconds with counter stain reagent, hematoxylin (blue).
11. Wash sections in ddH_2O for 5 min x 2.

Dehydrate/ Mounting:

(This method should only be used if the chromogen substrate is alcohol insoluble (e.g. Vector Red, DAB))

1. Wash slides 2X in 80% alcohol for 1 min at room temperature.
2. Wash slides 2X in 95% alcohol for 1 min at room temperature.
3. Wash slides 3X in xylene for 1 min at room temperature.
4. Mount the sections with coverslip.