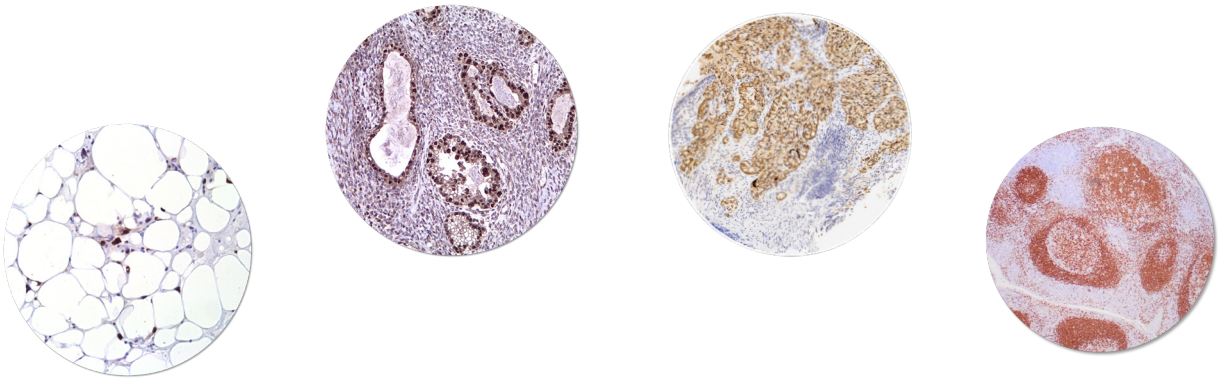
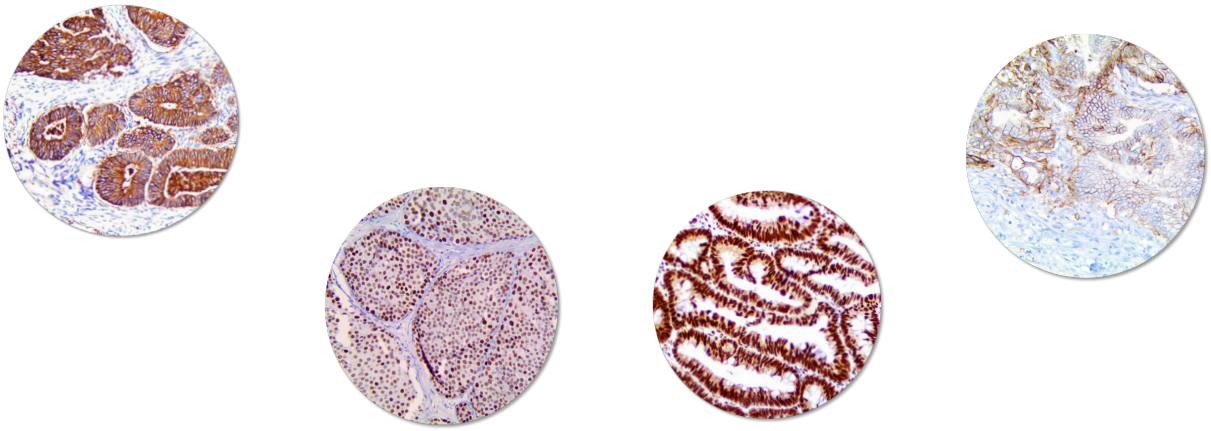
DIAGOMICS
ONCOLOGY EXPERT



INSTRUCTIONS FOR MANUAL IHC



IHC protocol

See our article on IHC on our website

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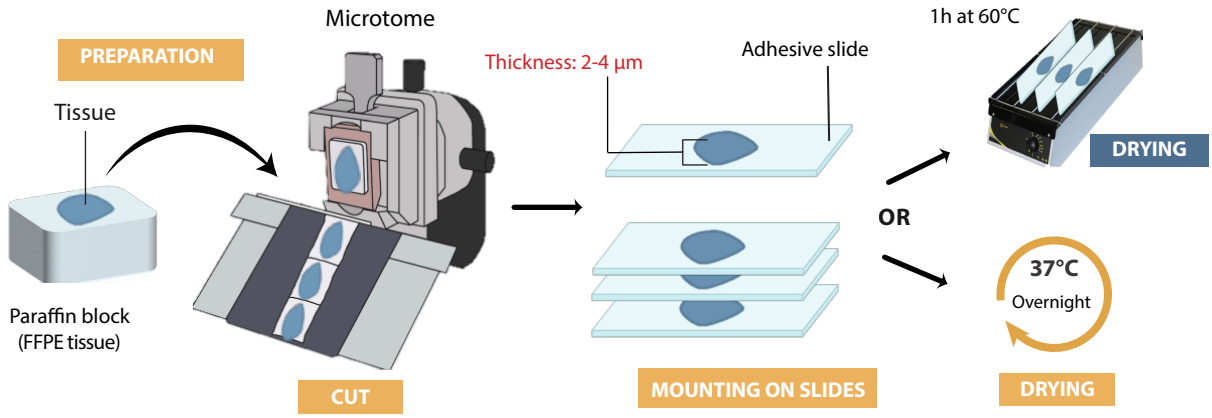
DIAGOMICS ONCOLOGY EXPERT

PROTOCOL : Preparation



- **Preparation of the paraffin block with the tissue**, to be subsequently cut with a microtome (thickness of 2-4 µm) and mounted to adhesive slides;
- **Incubation** overnight at 37°C or for 1 hour at 60°C;
- **Preparation of a series of decreasing alcohols** under an extraction hood;
- Solutions are freshly prepared at least 1 time/ week (e.g. after 200 sections);

  Denatured ethanol (≥ 99,8%) or alternatively isopropanol is diluted with demineralized water (96:4 for 96%, 80:20 for 80% and 70:30 for 70%).

Note: The hood must be turned on as soon as the lids of the cuvettes containing xylene or ethanol are opened.

PROTOCOL : Heating and deparaffinization

This step of deparaffinization is used to remove **paraffin from the tissue sections on the slides** and will be followed by **endogenous peroxidases blocking step** to reduce non-specific background staining.

1. **Heat the slide on a hotplate (10 min, 60°C) or in an incubator (1h30 or 2h30 at 58°C).**
2. **Put** the slide in **xylene** (e.g. 3x10 min, under the hood).
3. **Dip** the slide in a **series of decreasing alcohols**, (e.g. under the hood, 3x100 % alcohol for 2 min et 1 x 96 %, 80 % et 70 %).
4. **Place** the slide in 3% **H2O2 or peroxide block** (e.g. 1 x 10 min, under the hood).

PROTOCOL : Pretraitement

This step to **unmask the epitopes** is performed according to the information in the data sheet/manual of the antibody. In the case of PIER (step 5b), the **application of a hydrophobic border** can be done with the **PAP pen** before heating and in the case of HIER (step 5) after heating.

5a **HIER (Heat Induced Epitope Retrieval) :**

Put the slide in the **pretreatment buffer in the Pressure cooker** (BioSB); **alternatively**, you can preheat **a water bath or a steamer** with the pretreatment buffer and put the slide in hot buffer (approx. 96°C) for 30-40 min and then permit to cool down for 10 min.

5b **PIER (Protease Induced Epitope Retrieval) :**

Drop **enzyme onto the sections** (depending on the size of the section, 3-4 drops or 150-200 µl) and **incubate** the slide **in a humid chamber** (5 min, RT).

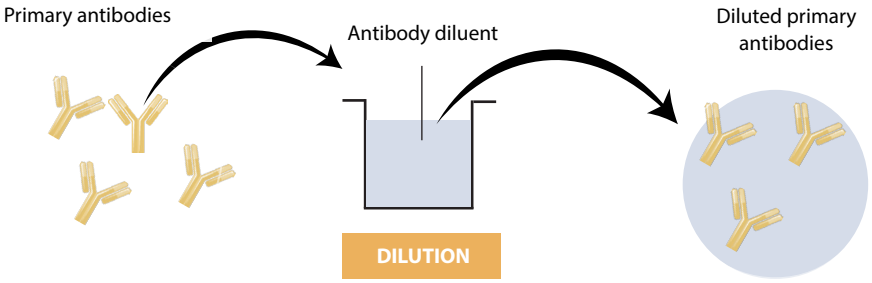
Note: From antibodies we market, three of them work best with enzymatic digestion: EGFR, Neuroblastoma and Collagen IV (BioSB).

6 **Rinse** the slide with **tap water** (at least 2 min).

7 **Put** the slide in **wash buffer** (default: TRIS wash buffer).

PROTOCOL : Primary antibodies

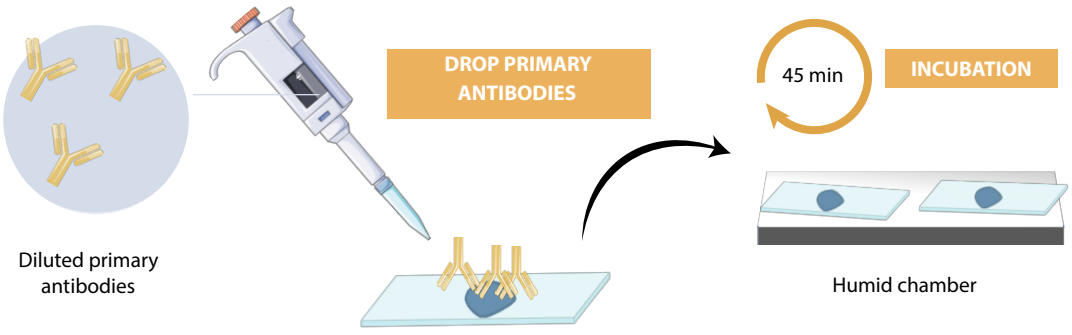
In this step the antibody is bound to the antigen in the following steps:



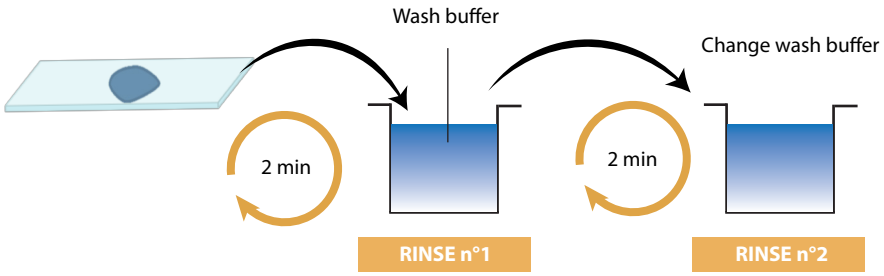
The primary antibody is diluted according to the information in the data sheet/instructions for use in the antibody diluent. If the antibody diluent already contains a blocking solution, the blocking step (usually necessary) to reduce non-specific binding is omitted.

8 Optional: **Apply** a **hydrophobic barrier around sections** on the slide with a **PAP pen**.
If the blocking solution is not present in the diluent, place the blocking solution (e.g. goat serum, BSA, commercial blocker...) and incubate 10-30 min at RT or ON at 4°C.

9 **Drop or pipet** the **primary antibodies solution** (note the dilution) onto the sections until the section is completely covered (depending on the size of the section, 3-4 drops or 150- 200 µl). This also applies to all following steps. **Incubate** the slide in a staining/humid chamber (45 min, RT, incubation time may vary depending on the antibody).



10 **Wash** the slide twice in **wash buffer** in a cuvette filled with wash buffer, rinse for 2 min, change wash buffer, rinse for 2 min.

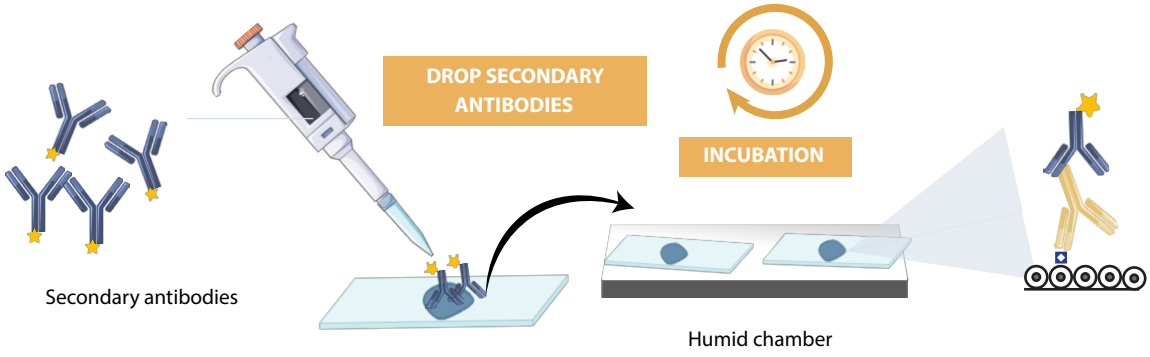


PROTOCOL : Detection

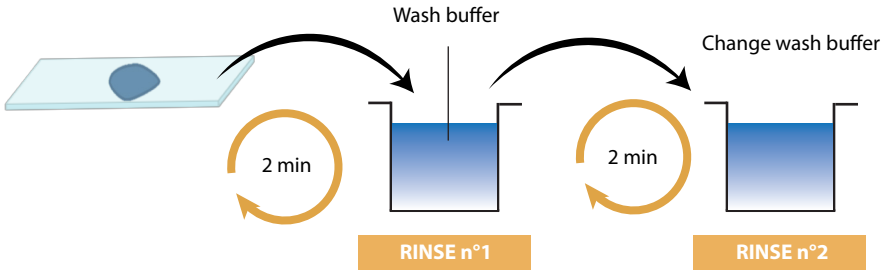
The following steps are designed **to detect antibody-antigen binding** using an appropriate detection system. Generally, two-step polymer systems are used according to the data sheet/operating instructions for use. For **signal amplification** you can refer to the protocol of the detection system. In a subsequent step, the corresponding **chromogen** (e.g. Permanent AP Red or DAB) is added, which **will be catalyzed by the enzyme** (AP, alkaline phosphatase or HRP, horseradish peroxidase) **to produce a colored, visible precipitate.**

11 **Remove excess wash buffer** from the slide.

12 **Drop or pipet detection solution onto the sections** and place slide in the staining/humid chamber (with time varying according to the detection system, RT).

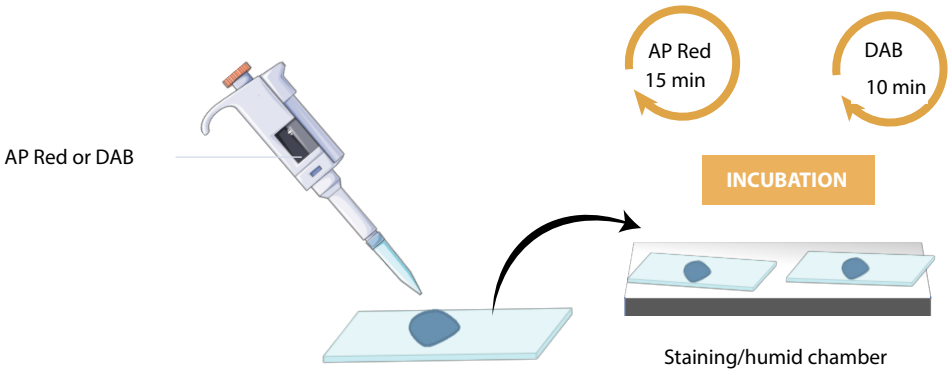


13 **Wash** the slide **twice in the cuvette filled with wash buffer**, rinse for 2 min, change wash buffer, rinse for 2 min.



14 **Remove excess wash buffer** from the slide.

15 **Apply chromogen** (as in the data sheet/instructions for use) onto the sections and place the slide in the **staining/humid chamber** (DAB 10 min, permanent AP red 15 min, RT).

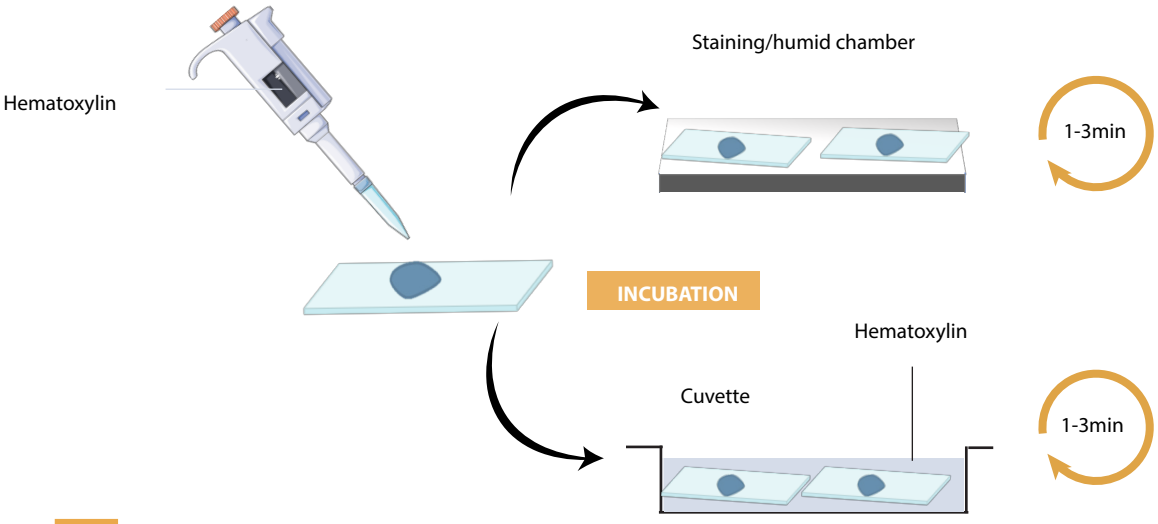


16 **Rinse** the slide thoroughly with **tap water** (at least 2 minutes, at room temperature).

PROTOCOL : Counterstaining

This step is usually used to visualize the tissue structure with **hematoxylin/haemalaun**. The hematoxylin is diluted 1:5 with distilled water (up to 1:10) and, if necessary, filtered before use.

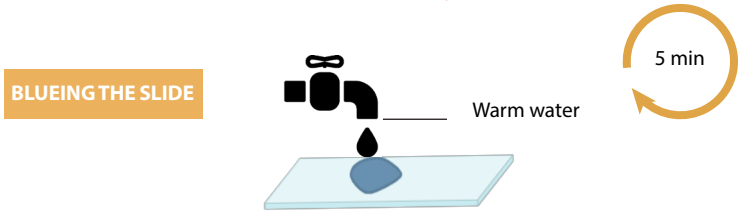
17 **Drop or pipet hematoxylin** (at the required dilution) onto the sections and place the slide in the **staining/humid chamber, or** set slide in a **cuvette with hematoxylin** (up to desired intensity for 1-3 min, RT).



18 **Wash** the slide briefly with **distilled water.**

19 **Blueing the slide** with **warm tap water** (usually about 5 min; until the violet hue has changed to blue color).

Except when using Permanent AP Red: Blue only in cold water, do not use warm water!



PROTOCOL : Mounting

In order **to fix the staining as well as to evaluate and archive the slides**, the sections are **covered with a coverslip using a mounting medium** (permanent or aqueous, depending on the chromogen).

20 If you use a **permanent mount the first step is to dehydrate the section** by performing **series of ascending alcohols** (70%, 80%, 96% under a hood).

21 **Then, set** the slides in **xylene** (2 x 1 min, under a hood).

22 **Mount the section** with coverslip using the mounting medium and allow to dry under a hood for at least 10 min.