In Situ Polymerase Chain Reaction—Imperiale Protocol

(Sources used include: Current Protocols (MJ Research's), as well as Nuovo, Strieter and Diamond Protocols.)
Abbreviations: RT = room temperature, '' = seconds, ' = minutes, λ = microliter, dH_2O = distilled water, diH_2O = deionized water, MM= master mix, Ab= antibody, α= anti-, DIG= digoxigenin, AP=alkaline phosphatase.

1. Refer to above sources if using fresh/frozen tissue. This protocol is for paraffin embedded tissues only.

2. Obtain 4-μm serial sections on silanized (AES-subbed) slides. Slides can be ordered from Sigma. Make sure the slides are numbered in order and that the microtome blade is sharp.

3. All solutions are made with diH_2O. Sterilize all solutions, glassware and disposable plastics. If glassware is not bakeable or autoclavable, store continuously in 10% bleach solution then rinse thoroughly with distilled water before use.

4. Prewarm 35mL of TBS in Coplin jar in 37°C water bath. Prewarm heat block (turned upside down) to 95°C.

5. **Deparaffinize and rehydrate** tissue sections with histology-grade xylenes and ethanol series. Change xylenes and replenish ethanol series regularly to get optimal results (ex every 20 slides). When not in use keep covered. Use fume hood—very volatile.

Xylenes-1	5'
Xylenes-2	5'
100%EtOH-1	3'
100%EtOH-2	3'
95%EtOH	1'
70%EtOH	1'
50%EtOH	1'

TBS for 5-10 min. (Can go to 20' if necessary.)

6. Add 10.5μL of Proteinase K stock to the prewarmed 35 mL of TBS in Coplin jar (final concentration 6μg/mL) . Invert to mix well.

7. **Unmask target sequence and destroy nucleases.** Add rehydrated slides to the proteinase K solution and let incubate 25' sharp at 37°C. Note that in an ideal world, this step is optimized for each tissue type and standardized method of fixation. Refer to above sources for target timepoint ranges.

8. **Inactivate PrK** by incubating slides flat (tissue section facing up) on the prewarmed upside down heat block, 2' at 95°C. Immediately immerse slides in room temperature TBS 10'', sterile diH_2O 10'' then 100%EtOH 10''. Let air dry in a dust free area. (I like to use a plastic 150mm cell culture dish.)

9. **Make PCR amplification reaction on ice.** Make sure individual components are completely thawed and well-mixed before addition to master mix. The Self-Seal (from **MJ Research**) is very viscous and must be inverted slowly 10 times before use. For prostate, sections are so big must use another slide rather than a coverslip to seal reaction chamber. This requires a minimum Master Mix volume of 75λ/section. Change gloves between primer sets and add the primers last.

Program "ERIN1"

- 1' @ 94°C
- 1' @94°C, 1' @50°C, 1' @72°C — 25 cycles
- 4°C soak to infinity

Component	Master Mix	Stock	Final
PCR diH_2O	--	--	To volume
10X PCR Buffer	10X	--	1X
$MgCl_2$	33X	50mM	1.5 mM
d NTP Mix	200X	20mM each	100μM each
DIG-dUTP	200X	1mM	5μM
BSA-V	25X	50mg/mL = 5%	0.2% or 2 mg/mL
Self Seal	2X	--	1X
Primer-forward	20X	25μM	1.25μM
Primer-reverse	20X	25μM	1.25μM
Platinum TaqPol	50X	5U/λ	0.1U/λ

10. Before you apply MM to tissue section, wipe down bench with 5% bleach and then 70%EtOH. Slides may prepared at room temperature on the precleaned bench. Handle negative controls first and positive controls last. Wipe down bench and change gloves between primer set master mixes.

11. **Apply 75λ to section of slide.** Aliquot slowly and do not try to expel every tiny last drop from the tip (introduces bubbles). Remove any bubbles that are introduced into the applied master mix. Gently "coverslip" the tissue section with another hydrophilic (AES-subbed) slide. With a fresh KimWipe, remove any amplification reaction that leaked out from between the slides. Change micropipette tips between each slide so that the 1X Self Seal does not dry within the tip.

12. Gently guide slides into that awesome PCR machine, and let it do its thing. The "ERIN1" Program is about 2.75 hrs long (block ramps very slowly). Now is a good time to take pictures of yesterday's expt.

13. After PCR, thaw an aliquot of 200mg/mL BSAV.

14. Immerse "coverslipped" slides in TBS at least 10 min. With a new razor blade gently pry apart the slide from its "coverslip." Discard used "coverslip" slides—never reuse.

15. **Block slides in 50mg/mL BSAV:TBS@RT for 30' with extremely gentle agitation.** Do not exceed "5" on the rocker. While blocking, make Ab dilution, prepare humid chamber and prewarm 35 mL of APSB in Coplin jar @ 37°C.

16. After blocking is finished, drain each section individually on a paper towel. Wipe away BSA:TBS from top of slide, staying clear of tissue. Apply 40-50λ of diluted Ab to section, making sure section is compleley covered. If blocking solution is wiped away thoroughly, there is NO NEED for a hydrophobic Pap pen which often bleeds over tissue section, preventing Ab access.

17. **Incubate with α-DIG AP Ab 1:100 in fresh CDB @RT in humid chamber**. Now is a good time to get the color reaction ready. The AP/NBT/BCIP color reaction is light-sensitive and must be executed in the dark to absolutely minimize nonspecific background. Move the following into the darkroom in anticipation of the color reaction: the light microscope, a timer, a p200 micropipetter and tips, an icebucket containing the stocks of NBT, BCIP and a Coplin jar full of prechilled dH_20 .

18. **Washes**: TBS, 10'@RT (twice) then cold APSB for at least 5' @RT.

19. When the slides are in the last (cold) APSB wash , add equimolar amounts of the NBT (140λ) and BCIP (116λ) stocks to the prewarmed APSB. If tissue of interest has endogenous alkaline phosphatase, add 0.8g of levamisole powder to the APSB to block. Cap and invert to mix.

20. **Add slides to color reaction**, and let reaction go no more than 5 minutes to keep background low. Positive signal from the AP conjugate is visible under the microscope after only 1-2 minutes.

21. **Stop color reaction** immediately by immersing slides 5' in the 0°C dH_20. (I use a 3.5 minute timepoint.)

22. **Counterstain** with Nuclear Fast Red (KPL's "Contrast Red") 2'@RT. Pour counterstain back into bottle when you are done--can be reused forever.

23. Immediately immerse slides in the 0°C dH_20 for 1' to **rinse** off excess counterstain. Using cold water will give you a larger window of time for the rinse.

24. **Drain** slides frosted end down in the white slide holder; let air dry at least an hour.

25. In hood, **coverslip** by dipping slides momentarily in xylenes, then placing a small drop of Permound over section (no bubbles allowed!). Gently lower coverslip over Permound drop, push out any bubbles introduced and rinse again in xylenes. Let air dry horizontally, and view.