

**GENERAL GUIDELINES:**

- Reactions volume depends on the sample size.
- Use best practices when pipetting to minimize reagents consumption.
- Centrifuge vials before pipetting.
- Select the Buffer C vial with the appropriate detection fluorophore for your microscope filter set.
- Completely defrost all buffer mixtures at room temperature, vortex well before use.
- Vortex and spin-down all enzymes (A, B and C) before use.
- Keep enzymes on ice or a frozen cold block.
- Wait to add enzymes until immediately prior to adding to the sample.
- Buffer C is a light-sensitive reagent. Always keep it protected from light.
- Remove excess washing buffer from samples before adding reagent.
- Do not allow slides/samples to dry.
- Preheat the humidity chamber before each step.
- Incubation times or temperatures other than those specified may give erroneous results.
- As with any product derived from biological sources, proper handling procedures should be used.
- Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
- Unused solutions should be disposed of according to local regulations.

**IMPORTANT:** Appropriate precautions should be taken to avoid antibodies cross-contamination. Cross-contamination is the main source of unspecific background due to the high sensitivity of the assay.

**Avoid bulk washing methods when multiple antibodies are used.**

**KIT COMPONENTS:****Box 1:**

Material	Amount	Storage*
Blocking Buffer (1x)	4000 µl	at +4 to +8°C  DO NOT FREEZE!!!
Primary Antibody Diluent (1x)	8000 µl	
Probe Diluent (1x)	4000 µl	
Probe 1	100 µl	
Probe 2	100 µl	

**Box 2:**

Material	Amount	Storage**
Buffer A (5x)	800 µl	at -25 to -15°C  PROTECT FROM LIGHT
Enzyme A1 (40x)	100 µl	
Buffer B (5x)	800 µl	
Enzyme B (40x)	100 µl	
Buffer C (5x) Atto 488	200 µl	
Buffer C (5x), TEX615	200 µl	
Buffer C (5x) Atto 647N	200 µl	
Enzyme C (40x)	100 µl	

\* When stored as directed, the product is stable at least for 3 months after receipt



1. Blocking
  - 1.1 Add **Blocking Buffer** (1x) to the entire sample area (approximately 40  $\mu$ l for each 1cm<sup>2</sup> area).
  - 1.2 Incubate for 60 min at +37 °C in a pre-heated humidity chamber.
2. Primary antibody incubation
  - 2.1 Use the provided **Primary Antibody Diluent** (1x) to dilute your primary antibody or antibodies.
  - 2.2 Decant the Blocking Buffer and add enough of your antibodies to cover the sample area.
  - 2.3 Incubate for 60 min at +37 °C or overnight at +4 °C in a humidity chamber.
  - 2.4 Decant the antibody solution and wash slides for 3x5 min with 1x TBS-T\*\* in a staining jar under gentle agitation.
3. Probe incubation
  - 3.1 Prepare the probes by diluting them in **Probe Diluent** (1x) (dilute 1:40 each).
  - 3.2 Add enough of the probes to cover the sample area.
  - 3.3 Incubate for 60 min at +37 °C in a pre-heated humidity chamber.
  - 3.4 Decant the solution and wash slides for 3x5 min with 1x TBS-T in a staining jar under gentle agitation.
4. Reaction A
  - 4.1 Start preparing **Reaction A** by diluting **Buffer A** (5x) 1:5 in water. Vortex and spin down.
  - 4.2 Add **Enzyme A** (dilute 1:40). Mix gently by pipetting and spin down.
  - 4.3 Add enough Reaction A to cover the sample area.
  - 4.4 Incubate for 60 min at +37 °C in a pre-heated humidity chamber.
  - 4.5 Decant the solution and wash slides for 2x3 min with 1x TBS-T in a staining jar under gentle agitation.
5. Reaction B
  - 5.1 Start preparing **Reaction B** by diluting **Buffer B** (5x) 1:5 in water. Vortex and spin down.
  - 5.2 Add **Enzyme B** (dilute 1:40). Mix gently by pipetting and spin down.
  - 5.3 Add enough Reaction B to cover the sample area.
  - 5.4 Incubate for 30 min at 37 °C in a pre-heated humidity chamber.
  - 5.5 Wash slides for 2x3 min with 1x TBS-T in a staining jar under gentle agitation.
6. Reaction C
  - 6.1 Select the **Buffer C** vial with the appropriate detection fluorophore for your microscope filter set. Do not use more than one **Buffer C** vial.
  - Protect from light**
    - 6.2 Start preparing **Reaction C** by diluting **Buffer C** (5x) 1:5 in water. Vortex and spin down.
    - 6.3 Add **Enzyme C** (dilute 1:40). Mix gently by pipetting and spin down.
    - 6.4 Add enough Reaction C to cover the sample area.
    - 6.5 Incubate for 90 min at +37 °C in a pre-heated humidity chamber.
    - 6.6 Decant the solution and wash slides for 2 min with 1x TBS in a staining jar under gentle agitation.
7. Nuclei staining
  - 7.1 Start preparing a Nuclei staining solution by diluting Dapi 1:1000 in 1xPBS. Vortex and spin down.
  - Protect from light**
    - 7.2 Decant wash buffer from the slides.
    - 7.3 Add enough Nuclei staining solution to cover the sample area.
    - 7.4 Incubate for 5 min at room temperature on the bench.
    - 7.5 Decant the solution and wash slides for 2x 10 min with 1x TBS in a staining jar under gentle agitation.
    - 7.6 Wash slides for 15 min with 0.1x TBS in a staining jar under gentle agitation.
8. Mounting
  - 8.1 Decant excess wash buffer from the slides.
  - 8.2 Mount the slides with a coverslip using a Fluoroshield anti-fade mounting medium.
  - Protect from light**
    - 8.3 Image your slides in fluorescence or confocal microscope, using 20x objective or higher and filter set for DAPI and a corresponding fluorophore (FITC, Texas Red, Cy5).

\*\*TBS-T (Tris-buffered saline supplemented with 0.05% Tween 20)