**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.
- NaveniFlex assay might be combined with traditional immunofluorescence techniques, providing that all primary antibodies are from different species and the fluorescence detection systems use different fluorophores.
- 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 | Article Number | Amount | Storage* |
|---|---|---|---|
| **Blocking Buffer (1x)** | NF.1.100.01 | 4000 µl | at +4 to +8°C |
| **Primary Antibody Diluent (1x)** | NF.1.100.02 | 8000 µl | |
| **Navenibody Diluent (1x)** | NF.1.100.03 | 4000 µl | |
| **Navenibody G1 (40x)** | NF.1.100.16 | 100 µl | DO NOT FREEZE!!! |
| **Navenibody R2 (40x)** | NF.1.100.07 | 100 µl | |

**Box 2:**

| Material | Article Number | Amount | Storage* |
|---|---|---|---|
| **Buffer A (5x)** | NF.2.100.08 | 800 µl | at -25 to -15°C |
| **Enzyme A1 (40x)** | NF.2.100.09 | 100 µl | |
| **Buffer B (5x)** | NF.2.100.10 | 800 µl | |
| **Enzyme B (40x)** | NF.2.100.11 | 100 µl | |
| **Buffer C (5x) Atto 488** | NF.2.100.13 | 800 µl | PROTECT FROM LIGHT |
| **Buffer C (5x), TEX615** | NF.2.100.12 | 800 µl | |
| **Buffer C (5x) Atto 647N** | NF.2.100.14 | 800 µl | |
| **Enzyme C (40x)** | NF.2.100.15 | 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 µl for each 1cm$^2$ 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 Navenibodies by diluting **Navenibody 1** and **Navenibody 2** in **Navenibody Diluent** (1x) (dilute 1:40 each). |
| | 3.2 | Add enough of the mixture to cover the sample area. |
| | 3.3 | Incubate for 60 min at +37 °C in a preheated 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<br><br>**Protect from light** | 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. |
| | 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 (not provided)<br>**Protect from light** | 7.1 | Start preparing a Nuclei staining solution by diluting Dapi 1:1000 in 1xPBS. Vortex and spin down. |
| | 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 (not provided)<br><br>**Protect from light** | 8.1 | Decant excess wash buffer from the slides. |
| | 8.2 | Mount the slides with a coverslip using an anti-fade mounting medium. |
| | 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 for Atto 488, Texas Red, or Cy5 for Atto 647, respectively) |

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