Navinci

Troubleshooting guide - NaveniFlex

High amount of background signal (in the technical control)

Insufficiently washed samples	• Increase the number of washes, the washing time, and/or the wash volume. • Use wash buffers indicated in the protocol. • Use a fresh wash solution. If it has become cloudy or there are any salt precipitates, prepare a new washing buffer.
Antibody crosscontamination	• Due to the high efficiency of detection, it is important to wash different antibody conditions separately to reduce cross-contamination between samples. • For NaveniFlex MM or RR: Try to wash samples where the primary antibody is omitted in separate washing jars. If that is not possible, use open droplet washing to avoid cross-contamination.
A too high concentration of the primary antibody	• Only for NaveniFlex RM: Titrate both primary antibodies individually while keeping the other constant and select the concentration for each of the antibodies that resulted in the highest signal-to-noise ratio.
Wrong fixation method	• Use fresh fixation reagents. • Use a fixation method recommended/optimized for the chosen antibodies. Recommendations for antibodies can be found in the datasheets.
Inadequate blocking	• Use the NaveniFlex blocking buffer for blocking and for diluting the primary antibody. • Cover the entire sample in the blocking solution. • Dilute the probes in the provided NaveniFlex probe diluent. • Increase blocking incubation time. • In certain cases, an alternative blocking protocol might be needed for a specific set of antibodies
Bad/dysfunctional probes	• Do not let the probes freeze. • Do not leave the probes at room temperature for extended periods. • Expired probes might not perform as expected.
Drying of sample	• Keep samples in a pre-heated humidity chamber during incubations. • Do not let samples dry out in between steps. To reduce the risk of drying, prepare the reaction mixtures before taking samples out of the washing buffer, and only remove a subset of your samples at a time.
Precipitate in buffer B	• Make sure that the buffer B is completely thawed. • The buffer can be left for 30 min at room temperature. If the precipitate has not been dissolved after vortexing, it can be heated in the palm of your hand until completely dissolved. • Always vortex reagents before use.
Non-RCA based fluorescent particles	• If growing cells: Wash off any culture medium before fixation. • Prepare fresh fixation reagents. • Use a fresh wash solution; if it has become cloudy or there are any salt precipitates, prepare a new washing buffer. • Ensure to wash the sample in 0.1x TBS before mounting. If the problem persists, dip the sample in pure water to remove any excess salt.
Unspecific primary antibodies	• If the background persists despite the critical determination of conditions (fixation, permeabilization, antibody titter, etc.), try an alternative primary antibody against the target(s).
Diffuse staining and/or autofluorescence	• Cell and tissue autofluorescence is often caused by the fixation procedure and can be reduced by investigating alternative fixation methods or by incubating with autofluorescence blocking reagents e.g. glycin. • Autoflurencent-like staining can be caused by unspecific binding of detection reagents in Reaction C. It can be removed by increasing the washing time after Reaction C and, in certain cases, by leaving the sample in 0.1x TBS overnight at 4°C. • Make sure to use only one of the Buffer C vials at the same time. To avoid potential autofluorescence in the two channels not used for detection. • Optimize the sample mounting as certain mounting media can cause haziness of the staining. Choosing a mounting medium without nuclei stain e.g. DAPI has shown to give clearer nuclei stain.

No or low amount of signal

Primary antibody from incorrect species	• Use primary antibodies suitable for the two probes. The two probes will bind an antibody from mouse and/or rabbit origin.
No or insufficient binding of primary antibodies	• Optimize sample preparation such as fixation, permeabilization, and antigen retrieval to ensure the binding of both primary antibodies. • Titrate the antibody(ies) and select the concentration that results in the highest signal. • For single protein detection, make sure that the antibody pair selected do not interfere with each other's epitope binding (only for NaveniFlex RM).

Incubation of reaction at the incorrect temperature	• Perform all incubations at the indicated temperatures, especially during the enzymatic steps (Reaction A, B, and C).
Excess wash buffer left on the sample	• Dilution of antibodies and/or enzymes by residual wash buffer will increase variability between samples/ experiments and reduce the efficiency. • Remove any excess wash buffer by aspiration or taping it off, before adding the reagent mixtures
Incorrect washing buffers	• Use wash buffers according to the protocol specification. Exchanging type of buffer can reduce efficiency. • Ensure the wash buffers are at room temperature.
Inefficient Reaction A, B or C	• Keep to the incubation times and temperature (37 °C) as stated in the protocol. • Remove any excess of wash buffer from the sample before adding the reagents. • Defrost the buffer at room temperature and vortex before use. • Always keep the enzyme at -20 °C for storage and on ice or a frozen cold block when pipetting to ensure enzyme activity. • Prepare Reaction A, B, and C fresh before use and add enzyme immediately before adding to the sample. • Incubate in a pre-heated humidity chamber.
Incompatible filter set during acquisition	• Use the appropriate filter set during image acquisition. • NaveniFlex has three different Buffer C vials. They contain different fluorophores with following wavelengths: Buffer C Atto 488: λex 504 nm; λem 521 nm, Buffer C TEX615: λex 596 nm; λem 613 nm and Buffer C Atto 647N: λex 644 nm; λem 667 nm. They are compatible with a filter set suitable e.g. FITC, TexasRed, and Cy5 fluorophores.

Poor imaging and analysis

Coalescing Signal	• Set exposure time to avoid over-exposure of any image. Never use autoexpose when acquiring images of the signal channel and use the same exposure time for all samples within an experiment. • One or both primary antibody concentrations could be too high. Consider titrating them. • Keep the incubation time (90 min) and temperature (37 °C) according to the protocol for reaction C, but for certain assays reducing the incubation time could reduce signals coalescing.
Autofluorescence	• Set exposure time to avoid over-expose of any image. Never use autoexpose when acquiring images of the signal channel. • Cell and tissue autofluorescence is often caused by the fixation procedure and can be reduced by investigating alternative fixation methods or by incubating with autofluorescence blocking reagents e.g. glycin. • For certain assays, increasing the incubation time for Reaction C can increase signal strength over autofluorescence. • Increase the number of washes after Reaction C. • For certain assays, leaving the sample in 0.1x TBS overnight at 4°C will reduce autofluorescence-like background from fluorophores present in Buffer C.

The variation between replications/experiments

Experimental set-up	• Different experimental set-ups e.g. a change in cell line/tissue, antibody, or cell treatment can introduce variation. To control for assay variability, always keep one of your conditions identical across all experiments.
Sample preparation	• Cell fixation, permeabilization, and cell density all affect the signal and should be constant between experiments. • Use the same tissue and tissue antigen retrieval protocol when assessing variation.
Deviation from the protocol	• Follow the protocol incubation times and temperatures. • If a deviation from the protocol is required, make the change consistently across all the experiments and change one experimental condition at a time.
Buffer mixing and pipetting errors	• Defrost buffers completely and allow to come to room temperature. Vortex reagents to ensure a homogeneous solution. • Use the same reagents across experiments in case of batch alteration due to suboptimal storage or contamination of specific tubes. • Use best practices when pipetting to minimize variation between experiments. • Immerse the pipette tip to the appropriate depth. Too much immersion causes enzymes to stick to the outside of the tip and increase the enzyme volume in the reaction.
Sample drying and insufficient removal of wash buffer	• Do not let the sample dry out in between steps. • Remove any wash buffer left before adding a reaction buffer to avoid dilution. • Work with fewer samples at a time if the two suggestions above are difficult to perform successfully.

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