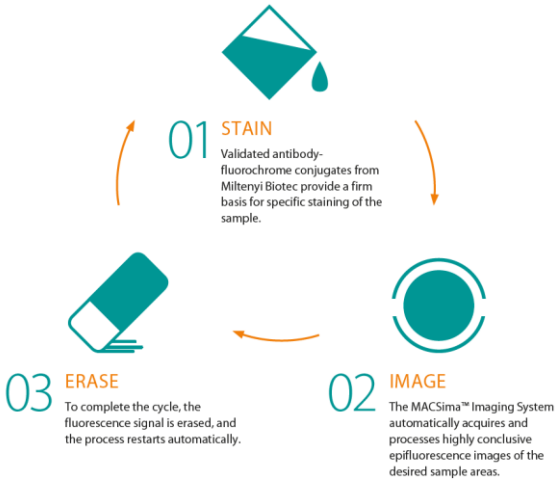
Antibody test protocol for the use in the MACSima™ Imaging Platform

The MICS technology

The MACSima Imaging System is a closed instrument made of a liquid handling system, a sample and reagent holder and an epifluorescence microscope, for the automated cyclic sample staining, imaging and signal erasure using the MICS technology (MACSima Imaging Cyclic Staining).
For more information:

miltenyibiotec.com/MACSima



Overview

To test whether a non-MICS-validated clone is functional for the MICS staining protocol this primary antibody can be tested using the following immunostaining protocol. This test aims to verify whether staining with the antibody of interest results in a specific signal after a 10 min incubation step to indicate functionality during MACSima experiments.

Both, fluorochrome-conjugated antibodies AND non-conjugated antibodies can be tested using this protocol.

Material

- Primary antibody to be tested for MICS (conjugated to fluorochrome such as FITC / PE OR pure/non-conjugated)
- (Optional) For test of pure/non-conjugated antibody, a secondary antibody conjugated to FITC / PE against the isotype of primary antibody is needed. These secondary antibodies have been successfully used:

Secondary antibody	Clone	Fluorochrome	Titer 1:x	Final concentration (µg/ml)	Provider	Order Number	Concentration (µg/ml)	Volume (ul)
mouse IgG1	X-56	PE	50		Miltenyi	130-119-585		200/1000
mouse IgG2ab	X-57	PE	50		Miltenyi	130-117-787		200
mouse IgG2c	Polyclonal	PE	500	1	Southern Biotech	1079-09	500	1000
mouse IgG3	SB76b	PE	100	1	Southern Biotech	1191-09L	100	2000
rat IgG1	RG11/39.2	PE	10		Miltenyi	130-104-833		1000
rat IgG2a	RG7/1.30	PE	50		Miltenyi	130-127-607		200
rat IgG2b	RG7/11.1.1	PE	10		Miltenyi	130-106-727		1000
rat IgG2c	R2C-23A3	PE	400	0,5	eBioscience	12-4816-82	200	500
rabbit IgG	2A9	PE/FITC	125	0,8	Southern Biotech	4090-09 PE 4090-02 FITC	100 (PE)/ 500 (FITC)	1000

- MACSima Running Buffer (PBS, EDTA, 0.09% sodium azide and BSA)
- MACSima System Buffer (PBS, EDTA, and 0.09% sodium azide)
- FcR Blocking Solution
- (Optional) DAPI Staining Solution
- Microscope slides
- Immunofluorescence microscope
- Sample of interest that expresses the marker of interest with known staining pattern fixed with either of the following fixation methods:
    - Formalin-fixed paraffin embedded (FFPE)
    - Paraformaldehyde (PFA)
    - Acetone
- Material for FFPE-fixation:
    - PT module (Thermo Fisher)
    - Cuvettes
    - Xylene
    - Ethanol 100%, 95%, 80%, 70%, 50%
    - Aqua dest.
    - Retrieval solution : TEC Buffer (Tris/EDTA/Citrate), pH9
- Material for PFA-fixation :
    - 4% PFA solution
- Material for acetone fixation
    - -20°C cold, freshly prepared acetone

Protocol

Process the sample according to the protocol of the fixation method of interest:

1. Formalin-fixed paraffin-embedded (FFPE) samples:
    a. Epitope retrieval protocol:
        i. Rehydrate Sections
        ii. Immerse the slides in xylene for 20 minutes.
        iii. Immerse the slides in 100% ethanol for 1 minute.
        iv. Immerse the slides in 95% ethanol for 1 minute.
        v. Immerse the slides in 80% ethanol for 1 minute.
        vi. Immerse the slides in 70% ethanol for 1 minute.
        vii. Immerse the slides in 50% ethanol for 1 minute.
        viii. Immerse the slides in deionized H_2O for 1 minute.
    b. Heat-induced Antigen Retrieval
        i. Immerse slides into preheated retrieval solution for 20 minutes by 98°C using the PT module.
    c. Stain the sample according to the staining protocol below

2. Frozen tissue slices: Paraformaldehyde (PFA) fixation
    a. Put frozen, non-fixed tissue slides in freshly-prepared 4% PFA solution for 10 minutes at room temperature.
    b. Tissue slides will be washed three times in MACSima System Buffer
    c. Stain the sample according to the Staining protocol below

3. Frozen tissue slices: Acetone fixation
    a. Put frozen, non-fixed tissue slides in -20°C cold, freshly prepared acetone for 10 minutes at -20°C.
    b. Air dry the sections at room temperature.
    c. Put sample into staining buffer.

4. Staining protocol

If the antibody supplier provides a recommended dilution for IHC / ICC in µg/ml or x:100, this is a good orientation for the test. If this is not provided, we suggest to test in a range of

- 1 µg/ml – 12.5 µg/ml (if concentration known)
  Or
- 1:50 – 1:150 (if concentration not known)

    a. Prepare a dilution series of the primary antibody in MACSima Running Buffer
    b. (Optional) Add FcR blocking reagent to the antibody solution
        i. Note: for REAfinity, REAlease or REAdye_lease antibodies, no FCR Blocking Solution is required

c. (Optional) DAPI Staining: Dilute DAPI Staining Solution in the antibody solution at 1µg/ml for tissue and adherent cells or at 5µg/ml for suspension cells.
d. Add the antibody solution to the sample and incubate 10 min at room temperature
e. Wash the sample 6 times with MACSima System Buffer

(Optional) Secondary antibody staining for detection of pure/non-conjugated antibodies:

a. Dilute the secondary antibody in MACSima Running Buffer
b. Add the antibody solution to the sample and incubate 10 min at room temperature
c. Wash the sample 6 times with MACSima System Buffer

5. Image acquisition

a. Image the sample without mounting in mounting medium
b. Acquire 3 different exposure times per channel
    i. FITC → 75, 300, 1200 ms
    (reference for fluorochrome-conjugated primary antibodies)
    ii. PE → 40, 160, 640 ms
    (reference for fluorochrome-conjugated primary antibodies)
c. Evaluate images for expected staining pattern and sufficient signal intensity
    i. Evaluate if staining signal reflects subcellular localization of target marker
    ii. Evaluate if staining pattern corresponds to published data: protein atlas data, scientific literature and expression databases
    iii. Choose the antibody concentration with the best signal-to-background ratio for use in the MACSima
        1. If an indirect IF staining was performed to evaluate the MICS-compatibility of a non-conjugated primary antibody, please note that the optimal concentration should be titrated after the conjugation to fluorochromes