Multiplexing Immunofluorescence (MX-IF)

Using complexes pre-formed from primary antibodies and secondary FluoTags

Multiplexing immunofluorescence allows researchers to stain a single sample with multiple primary antibodies originating from the same species, even if they have identical isotype. Stainings can, e.g., be performed with multiple monoclonal mouse IgG1 kappa primary antibodies or with multiple polyclonal antibodies raised in rabbit at the same time.

NanoTag Biotechnologies offers a choice of secondary reagents that are validated for multiplexing applications:

FluoTag®-X2 anti-Mouse IgG Kappa Light Chain	**N1202**
FluoTag®-X2 anti-Mouse IgG1	**N2002**
FluoTag®-X2 anti-Mouse IgG2	**N2702**
FluoTag®-X2 anti-Rabbit IgG	**N2402**

All secondary FluoTag®-X2 reagents offered by NanoTag are based on monovalent single-domain antibodies ("nanobodies") that are site-specifically coupled to two fluorophores per nanobody. They are supplied as solutions containing 5 µM nanobody (10 µM fluorophore).

Important general remarks

For multiplexing applications, all primary antibodies need to be well characterized. The stoichiometry between primary antibody and the secondary FluoTag reagent is critical. Therefore, the concentration of the primary antibody needs to be precisely known.

- Monoclonal mouse antibodies are generally provided by the supplier at a defined concentration. In case cell culture supernatants are used or the concentration of the antibody is unknown, it might be required to purify the antibody e.g. via Protein G to remove serum proteins or other additives before quantification.
- For polyclonal rabbit antibodies, an affinity purification step is mandatory in order to remove serum proteins and antibodies not recognizing the target protein

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I. Components required

a) Multiplexing with primary mouse monoclonal antibodies

- Primary mouse monoclonal antibodies (not supplied)
- Fluorescently labeled secondary FluoTags matching the isotype of the primary antibodies used
  FluoTag-X2 anti-Mouse Kappa Light Chain (N1202)
  FluoTag-X2 anti-Mouse IgG1 (N2002)
  FluoTag-X2 anti-Mouse IgG2 (N2702)
- Multiplexing Blocker Mouse (MXB-M) (optional)

It is ideal to use the same type of FluoTag reagent for all mouse antibodies in a given mixture. If, for any reason, different FluoTag reagents are combined (e.g. FluoTag-X2 anti-Mouse Kappa Light Chain and FluoTag-X2 anti-Mouse IgG1) it is mandatory to include the multiplexing blocker.

b) Multiplexing with affinity-purified primary rabbit antibodies

- Affinity-purified primary antibodies raised in rabbit (not supplied)
- Fluorescently labeled secondary FluoTags
  FluoTag-X2 anti-Rabbit IgG (N2402)
- Multiplexing Blocker Rabbit (MXB-R) (optional)

II. Protocol

1. Complex formation

- Determine the amount of purified primary antibody required for your experiment. If unknown, start with 1 µg of each primary antibody.
- To saturate all binding sites, combine in separate reaction tubes each of your primary antibodies with an appropriate labeled secondary FluoTag.

  Use 20 pmol (4 µL of secondary reagent as supplied by NanoTag) of secondary FluoTag per µg primary antibody. This will result in a 50% excess of labeled nanobody over available binding sites.
- Add 20 µL PBS per µg of primary antibody
- Incubate for 20 min at room temperature with moderate shaking.

2. Optional blocking step

The optional blocking step will minimize potential issues arising from a slow exchange of the secondary reagents between different primary antibodies ("hopping"). Hopping is generally negligible for short incubation and washing periods (total time >12 h at room temperature or >24 h at 4°C). For protocols requiring extended incubation or washing steps, the optional blocking step is recommended.

- Add 10 µL of Multiplexing Blocker (**MXB-M** or **MXB-R**) per µg of primary antibody.
- Incubate 5 min at room temperature with moderate shaking.

3. Preparing the final staining solution

- Mix and dilute all pre-formed complexes to reach the respective final concentrations required for your experiment. A recommended dilution buffer for immunofluorescence applications is PBS, 0.1 % Triton-X100, 1 % BSA
- Immediately proceed to step 4

4. Staining procedure

The optimal staining procedure may depend on the sample type and the primary antibodies used. We can therefore only give a starting protocol here that generally works well for standard immunofluorescence (IF) applications on fixed cultured cells. A more detailed IF protocol can be found at https://nano-tag.com/protocols.

Short protocol:

- Stain your fixed and permeabilized sample for 1 h at RT protected from light.
- Wash 3 x for 5 min with PBS
- Rinse samples with water before mounting to remove excess salt.
- Mount and image sample

III. Hints for optimization

- Test single stainings (without multiplexing) in order to determine the minimal concentration of primary secondary mixture required to give adequate staining with minimal background.
- Use the lowest concentrations of primary antibody and secondary tools required for optimal stainings. High concentrations of primary antibodies and/or large excess of secondary tools may result in non-specific background staining.
- Avoid long incubation and/or extensive washing steps whenever possible.
- For extended incubation steps include an appropriate multiplexing blocker reagent.
- It is ideal to image the samples shortly after mounting.

  An additional post-fixation step is recommended when encountering problems with fluorophore hopping after mounting the sample.

For further information concerning this protocol please contact us at info@nano-tag.com