

Protocol

immunoMUSE[®] Amplify Kit

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immunoMUSE® Amplify Kit

Product description

ImmunoMUSE® Amplify kits are designed to perform high sensitivity single or multiplex immunofluorescence stainings of various fixed (FFPE, adherent cells) samples. Our MUSE® nanotechnology enables the use of virtually any primary antibody (BSA- and azide-free) with high signal amplification and does not require secondary antibodies. Our kits are available as 1-plex, 2-plex and 4-plex solutions.

Caution: Do not mix components from different kits, as this can negatively affect the staining outcome and may result in staining failure.

Product contents

The contents of the ImmunoMUSE® Amplify kits are the following:

Kit type (Cat.No.)	Product Name	Qty and vial type	Storage
IMK1X05-	ImmunoMUSE Antibody buffer	1 unit, 1.2mL in a 2 mL screw cap tube	2-8°C Protect from light and don't freeze
	MUSE-A	1 unit, 300µL in a 0.5mL screw cap tube	
	MUSE-B	1 unit, 300µL in a 0.5mL screw cap tube	
	MUSE-C	1 unit, 300µL in a 0.5mL screw cap tube	
	MUSE-D	1 unit, 300µL in a 0.5mL screw cap tube	
IMK1X25- IMK2x25- IMK4X25-	ImmunoMUSE Antibody buffer	1 unit, 5.5mL in a 15mL screw cap bottle	2-8°C Protect from light and don't freeze
	MUSE-A	1 unit, 1.3 mL in a dropper bottle	
	MUSE-B	1 unit, 1.3 mL in a dropper bottle	
	MUSE-C	1 unit, 1.3 mL in a dropper bottle	

	MUSE-D	1 unit, 1.3 mL in a dropper bottle	
IMK1X50- IMK2x50- IMK4X50-	ImmunoMUSE Antibody buffer	1 unit, 11 mL in a 15mL screw cap bottle	2-8°C Protect from light and don't freeze
	MUSE-A	1 unit, 2.6 mL in a dropper bottle	
	MUSE-B	1 unit, 2.6 mL in a dropper bottle	
	MUSE-C	1 unit, 2.6 mL in a dropper bottle	
	MUSE-D	1 unit, 2.6 mL in a dropper bottle	

Additional materials required

Equipment

- Oven or incubator set at 60°C
- Optional: Fume hood for volatile solutions during pretreatment
- Steamer or Antigen retrieval chamber for HIER
- Fridge or cold room set at 4°C
- Widefield or confocal microscope

Consumables

- Slide staining chamber
- Hydrophobic barrier Pap Pen (VectorLabs ImmEdge pen is recommended)
- Coplin jars and glass slide holder (optional)
- Paper towels
- Glass coverslips

Reagents

- Samples for the staining
- MUSE®-conjugated IgG antibody (-ies)
- Deparaffinization reagent (such as Histo Clear II)
- Absolute Ethanol solution (100%)
- Antigen retrieval solution (such as Universal HIER buffer)
- Double Distilled/ MiliQ water
- 2XSSCT buffer (2XSSC + 0.1% Tween-20)
- PBST buffer (PBS + 0.1% Tween-20)

- Hydrophobic barrier Pap Pen (VectorLabs ImmEdge pen is recommended)
- DAPI nuclear stain in 2XSSCT buffer (Optional)
- Mounting medium (Prolong Gold Antifade is highly recommended)

Step 1. Sample pretreatment

You should be able to follow your preferred sample pretreatment. The instructions below can be adjusted based on your experience.

1. Bake slides **60 minutes at 60°C**.
2. Deparaffinize slides:
 - i. **3 X 5 minutes** in **HistoClear II**.
 - ii. **3 X 5 minutes** in **100% EtOH**.
3. Wash for **2 X 3 minutes**, gently shaking the slides in staining jars with fresh **ddH₂O**.
4. Dry slide by blotting edges on a paper towel.
5. Perform target antigen retrieval with **HIER solution** for **15-30 minutes** (depending on your antigen) *or an appropriate antigen retrieval step for your sample and antigen*.
6. After the incubation, dip the hot container containing the slides in cold water and leave it to cool down for **10 minutes**.
7. Transfer slides to **ddH₂O** for washing **2 X 1 minute**.
8. Dry slide by blotting edges on a paper towel.
9. Draw a barrier around tissue with an hydrophobic barrier PAP pen, let dry (**~ 5 minutes**). Make sure to add a drop of **PBST** on the tissue so that it does not dry out.
10. Rinse slides in staining jars with **PBST** for **2 X 2 minutes**.
11. Proceed with antibody blocking.

Step 2. Antibody incubation

i. Blocking

1. Remove last **PBST** rinse by blotting the slide edges on a paper towel.
2. Add 100-200 μL of **ImmunoMUSE antibody buffer**.
3. Incubate for **10 minutes** at room temperature in a **humidified staining chamber**.

ii. Antibody incubation

4. For each sample, prepare an antibody solution *by diluting your MUSE antibody-conjugate into **ImmunoMUSE antibody buffer**. (Choose an appropriate dilution for your antibody, we recommend to start with a 1/400 dilution. MUSE can enable further dilutions which can be tried at later steps.)*
5. Remove the **MUSE antibody buffer** from the blocking step by blotting edges on a paper towel.
6. Add the **antibody solution** to the sample.
7. Incubate according to the requirements of your antibody and sample *(e.g., **overnight at 4°C** in a humidified chamber or **2 hours at room temperature**.)*

iii. Antibody washes

8. Remove the **antibody solution** by blotting edges on a paper towel.
9. Wash **3 X 10 minutes** in **PBST** at room temperature in a staining jar.

Step 3. MUSE® Amplification

The following steps can be done on a normal lab bench at room temperature. Use a humidified chamber to minimize evaporation.

i. Primary MUSE® amplification

1. Remove **PBST** by blotting edges on a paper towel.
2. Rinse slides in **2XSSCT** for **2-5 minutes**.

3. Remove as much as possible of the excess **2XSSCT** by blotting the slide edges on a paper towel.
4. Add **1 drop** of **MUSE-A** and **1 drop** of **MUSE-B** to the sample (1 to 1 ratio of MUSE-A and MUSE-B solution).
5. Incubate for **1-2 hours** at room temperature **in a humidified staining chamber**.

The volumes and incubation time are established for 4 µm FFPE sections. Bigger volumes and longer incubation times are needed for thicker sections or whole mount organs.

The degree of signal enhancement depends on the duration of the incubation. Keep the incubation times constant to maintain similar enhancement across experiments, if necessary.

ii. Primary amplification washes

1. Remove the **primary MUSE® amplification solution**.
2. Rinse with gentle shaking in a staining jar with **2XSSCT** at room temperature for **5 X 2 minutes**.

iii. Secondary MUSE® amplification



From now on the samples should be protected from light!

1. Remove the **2XSSCT** from the last wash.
2. Add **1 drop** of **MUSE-C** and **1 drop** of **MUSE-D** to the sample (1 to 1 ratio of MUSE-C and MUSE-D solution).
3. Incubate for **1-2 hours** at room temperature **in a humidified staining chamber**.

The volumes and incubation time are established for 4 µm FFPE sections. Bigger volumes and longer incubation times are needed for thicker sections or whole mount organs.

The degree of signal enhancement depends on the duration of the incubation. Keep the incubation times constant to maintain similar enhancement across experiments, if necessary.

iv. Secondary amplification washes and DAPI staining



Keep the samples protected from light.

1. Remove the secondary MUSE® amplification solution.
2. Rinse with gentle shaking in a staining jar with **2XSSCT** at room temperature for **2 X 2 minutes**.
3. Incubate the sample with **1X DAPI in 2XSSCT** for **5 minutes** in a staining jar.
4. Rinse with gentle shaking in a staining jar with **2XSSCT** at room temperature for **2 X 2 minutes**.
5. Rinse with gentle shaking in a staining jar with **2XSSC (no Tween-20)** at room temperature for **2 minutes**.

Step 4. Mounting and Imaging



Below is an example protocol for Prolong Gold Antifade. If you would like to perform a fresh mount, we recommend using 2XSSC and imaging immediately. The mounting reagents often contain harmful volatile components, please read the manufacturer's instructions and manipulate appropriately.

1. Remove as much as possible from the **2XSSCT** from last wash using a folded paper towel without touching the sample.
2. Add a drop of **Prolong Gold Antifade**. Let the drop flatten and then add a coverslip. Absorb any excess liquid using a folded paper towel and touch the side of the coverslip.
3. Let the **Prolong Gold Antifade** set overnight at 4°C in a dry environment. Keep samples protected from light.
4. Ideally, image the samples the next day or shortly after.

Note: We have some evidence that the fluorescent signal is stable for 3-4 weeks in ProLong Gold but we cannot guarantee it for all markers.

Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
<i>No signal</i>	<ul style="list-style-type: none"> • Sample/Tissue degradation • Poor antigen retrieval • MUSE® Reagents omitted • Different wash buffer than 2XSSCT used • Wrong mounting media • Wrong filter or imaging settings 	<ul style="list-style-type: none"> • Check that the chosen HIER buffer is suitable for your antibody • Verify that all reagents were added in the correct order (First A+B and then C+D) • Execute washing steps with 2XSSCT • Use an antifade mountant compatible with the fluorophore(s) • Confirm microscope settings and channel assignment
<i>Weak target staining</i>	<ul style="list-style-type: none"> • Over-fixation or poor antigen retrieval (epitope damage) • Antibody concentration too low • Short incubation time • Antibody not well suited for IF • Different wash buffer than 2XSSCT used 	<ul style="list-style-type: none"> • Optimize fixation and retrieval conditions • Optimize antibody concentration or extend incubation time; conjugation of suitable MUSE® activator for low-abundant target • Switch to a validated IF antibody • Execute washing steps with 2XSSCT
<i>Strong background staining</i>	<ul style="list-style-type: none"> • Poor deparaffinization for FFPE sections • Antibody concentration is too high • Performed longer incubation times for amplification • Inadequate washing between MUSE® steps • Tissue autofluorescence (e.g. necrotic tissue, blood vessels, connective tissue) 	<ul style="list-style-type: none"> • Make sure to use fresh deparaffinization solution • Titrate conjugated MUSE® antibodies • Follow the incubation time of 1.5h per amplification step; Longer amplification can lead to increased background • Increase wash number, duration and stringency • Change fluorophore or include proper controls for image post-processing
<i>Uneven staining - Edge effects</i>	<ul style="list-style-type: none"> • Sample dried out during staining • Inconsistent reagent distribution 	<ul style="list-style-type: none"> • Keep samples moist by covering after deparaffinization; use a humidified chamber

	<ul style="list-style-type: none"> • Inconsistent fixation of the sample • Inadequate permeabilization in thicker sections • Section thickness variability 	<ul style="list-style-type: none"> • Ensure complete coverage of the sample with enough staining solution • Standardized fixation time and conditions • Optimize permeabilization condition for the tissue type • Cut sections as uniformly as possible
Channel crosstalk/ Panel imbalance	<ul style="list-style-type: none"> • Spectral overlap of fluorophores due to panel imbalance • Antibody concentration too high • Amplification of highly abundant target • Imaging settings too broad • 	<ul style="list-style-type: none"> • Follow the MUSE® conjugation scheme based on target abundance (see conjugation protocol) • Titrate Antibody to avoid spectral shifts • Narrow acquisition windows or use sequential imaging
Photobleaching and signal fade	<ul style="list-style-type: none"> • Prolonged light exposure during staining and /or imaging • High illumination intensity and repeated imaging of the same field • No antifade protection 	<ul style="list-style-type: none"> • Minimize light exposure and reduce excitation intensity • Limit repeated scans • Mount samples in an antifade medium
Poor reproducibility	<ul style="list-style-type: none"> • Variable tissue, staining and imaging conditions 	<ul style="list-style-type: none"> • Standardize incubation times, washes, and acquisition settings
MUSE® dots background staining Diffuse background signal may be distributed uniformly throughout the tissue and become detectable when the specific target signal is weak/absent. They may also appear at low intensity in negative controls, such as no-antibody controls. This nonspecific background should remain substantially lower in intensity than the specific target-associated staining.	<ul style="list-style-type: none"> • Stickiness of the amplifiers • Low signal of target 	<ul style="list-style-type: none"> • Increase antibody concentration • Optimize microscope settings

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