Protocols

immunoMUSE®Activate Kit

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immunoMUSE® Activate Kit

Product description

The immunoMUSE® Activate Kit enables selective attachment of an azide substituent to the heavy chains of an unlabeled IgG antibody. This targeted minor modification preserves the antibody's antigen-binding regions, ensuring they remain fully functional for recognizing the intended target. The resulting azide-modified antibody can then be covalently conjugated to the MUSE® Activator provided in the kit.
Each kit includes enough reagents to carry out azide modification reactions using 100 µg of whole IgG. It also provides antibody concentrators for purification and concentration at each stage of the conjugation workflow. We offer customized solutions for conjugating larger amounts of antibodies.

Product contents

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

Kit type (Cat.No.)	Product Name	Qty and vial type	Storage
IMK1	20× TRIS Buffer pH 7.0 (C1)	1 unit, 1.8mL in 2 mL screw cap tube (red lid)	2-8°C Protect from light and don't freeze
	Small Concentrator (0.5 mL) (C2)	1 unit	
	Collection tube (C3)	1 unit	
	Enzyme Solution (C4)	1 unit, 20µL in a 0.5mL screw cap tube (green lid)	
	Azide Connector (C5)	1 unit, 140µg in a 0.5mL screw cap tube (blue lid)	
	Buffer Complement (C6)	1 unit, 300µL in a 0.5mL screw cap tube (purple lid)	
	Large Concentrators (5 mL) (C7 + C9)	2 units	
	MUSE Activator S01 (C8)	5µL in 0.5mL screw cap tube	
IMK2	20× TRIS Buffer pH 7.0 (C1)	2 units, 1.8mL in 2 mL screw cap tubes (red lids)	2-8°C Protect from light and don't freeze
	Small Concentrator (0.5 mL) (C2)	2 units	
	Collection tube (C3)	2 units	

	Enzyme Solution (C4)	2 units, 20µL in 0.5mL screw cap tubes (green lids)	
	Azide Connector (C5)	2 units, 140µg in 0.5mL screw cap tubes (blue lids)	
	Buffer Complement (C6)	2 units, 300µL in 0.5mL screw cap tubes (purple lids)	
	Large Concentrators (5 mL) (C7 + C9)	4 units	
	MUSE Activators S01 and S03 (C8.1 and C8.3)	2 units, 5µL in 0.5mL screw cap tubes	
IMK4	20× TRIS Buffer pH 7.0 (C1)	4 units, 1.8mL in 2 mL screw cap tubes (red lids)	2-8°C Protect from light and don't freeze
	Small Concentrator (0.5 mL) (C2)	4 units	
	Collection tube (C3)	4 units	
	Enzyme Solution (C4)	4 units, 20µL in 0.5mL screw cap tubes (green lids)	
	Azide Connector (C5)	4 units, 140µg in 0.5mL screw cap tubes (blue lids)	
	Buffer Complement (C6)	4 units, 300µL in 0.5mL screw cap tubes (purple lids)	
	Large Concentrators (5 mL) (C7 + C9)	8 units	
	MUSE Activator S01-S04 (C8.1-C8.4)	4 units, 5µL in 0.5mL screw cap tubes	

Necessary material not provided

Equipment

- Centrifuge for 1.5 mL centrifuge tubes
- Centrifuge for 25 mL centrifuge tubes
- Heating block or incubator at 37 °C
- Vortex
- Nanodrop or similar to define antibody concentration

Consumables and Reagents

- 100 ug of IgG antibody, free of BSA and/or azide
- Double distilled (ddH_2O) or MiliQ water
- 1× PBS
- 1.5 mL reaction tubes
- Aluminum foil
- Parafilm™

Step 1. Antibody Concentration and/or Buffer Exchange

Carry out the antibody concentration and buffer exchange step if:
• The antibody concentration is below 2 mg/mL, and/or
• The antibody is in a phosphate-based buffer (e.g., PBS), and/or
• The antibody is in a buffer that contains azide, and/or
• The antibody is in a buffer that contains glycerol.

Before beginning, briefly centrifuge all tubes containing enzymes and substrates to collect the contents at the bottom.

Wash the antibody concentrator

1. Add 500 µL of ddH_2O to the small antibody concentrator (C2) and close the lid.
2. Centrifuge at 5,000 × g for 6 minutes.
   Note: Position the concentrator so that the strap of the lid and one membrane panel of the concentrator faces the center of the rotor.
3. Discard the flowthrough.

Concentrate the antibody and perform buffer exchange

1. Prepare 2 mL of 1× TRIS buffer (pH 7.0) for each 100 µg antibody sample by mixing 0.1 mL of 20× TRIS buffer (C1) with 1.9 mL of ddH_2O.
2. Add enough volume of antibody solution containing 100 µg of antibody to the small antibody concentrator.
3. Bring the total volume to 500 µL using 1× TRIS buffer.

   Centrifuge at 5,000 × g for 6 minutes.
   Note: Position the concentrator so that the strap of the lid and one membrane panel of the concentrator faces the center of the rotor.
4. Check the retentate volume; it should be ≤50 µL. If it exceeds 50 µL, centrifuge again in 3-minute intervals until volume is reduced.

5. Discard the flowthrough.
6. Add 450 µL of 1× TRIS buffer to the small antibody concentrator and centrifuge again at 5,000 × g for 6 minutes.
   Note: Position the concentrator so that the strap of the lid and one membrane panel of the concentrator faces the center of the rotor. If needed, repeat centrifugation in 3-minute intervals until the retentate volume is ≤50 µL.
7. Carefully invert the small antibody concentrator (C2) into the fresh collection tube (C3).
   Note: Flip the concentrator bottom-up, so that the opening of the concentrator is facing the inside of the collection tube. Make sure not to touch or damage the membrane during this step. Switch gloves between antibodies to avoid cross-contamination.
8. Centrifuge for 3 minutes at 1000 × g to retrieve the concentrated antibody. After collection, the collection tube should contain approximately 50 µL of concentrated antibody.

Step 2. Carbohydrate Modification of the Antibody and Azide Attachment

1. Adjust the antibody solution to a final volume of 50 µL using 1× TRIS buffer.
2. For each antibody prepare the azide modification mixture by adding the following to the tube containing Azide Connector (C5):
   - 25 µL ddH_2O
   - 5 µL of 20× TRIS
   - 10 µL Buffer Complement (C6)
3. Vortex to mix, then add the following reagents to the azide modification mixture:
   - 50 µL of the buffer-exchanged antibody
   - 10 µL of Enzyme Solution (C4)
4. Mix thoroughly and briefly centrifuge the tube.
5. Seal the tube cap with Parafilm™ (or an equivalent sealing film) and incubate overnight at 37 °C in a heating block or incubator.

   Optional: Cover the heating block with aluminum foil for a constant temperature.

Step 3. Purification and Concentration of the Azide-Modified Antibody

This step removes excess azide connector substrate.
You may also use TBS or other phosphate-free buffers for purification and collection of the modified antibody. 20× TRIS buffer, pH 7.0 is provided for your convenience.

1. Prepare 10 mL of 1× TRIS for each 100 µg antibody modification by mixing 500 µL of 20× TRIS buffer with 9.5 mL of ddH_2O.
2. Add 1 mL of 1× TRIS to the large antibody concentrator (C7).
3. Centrifuge at 3,000 × g for 6 minutes, ensuring that one membrane panel of the concentrator faces away from the center of the rotor.
4. Discard the flowthrough.
5. First add 2 mL of 1× TRIS and then transfer antibody solution to the large antibody concentrator.
6. Continue centrifugation in 3 minutes steps or until volume is reduced. Discard flowthrough.
7. Centrifuge at 3,000 × g for 6 minutes, ensure retained volume is 0.1 mL or less.
8. Add 2 mL 1× TRIS buffer to antibody concentrator, repeat centrifugation.
9. Discard flowthrough and repeat two more times.
10. Collect the azide-modified antibody from the concentrator with a pipette and transfer into a 1.5 mL reaction tube.
    Ensure to recover the entire volume using smaller pipette tips!
11. Optional: Determine the antibody concentration by measuring A280 (with A280 at 1.4 = 1 mg/mL) with a Nanodrop to evaluate loss of material.

Step 4. Attachment of MUSE® activator to azide-modified antibody

1. Transfer the azide-modified antibody into the tube containing MUSE activator (C8).
2. Mix carefully and briefly centrifuge the tube.

3. Following a room temperature incubation for 15 – 30 minutes, keep the mixture at 4 °C for 66-72 hours (2.5 days).

   For 2-plex and 4-plex: Conjugate the antibodies according to their target abundance following this scheme:

Target abundance	IMK2 (2-plex kit)	IMK4 (4-plex kit)
	MUSE activator S03	MUSE activator S03
	MUSE activator S01	MUSE activator S04
		MUSE activator S02
		MUSE activator S01

Step 5. Purification and concentration of MUSE®-conjugated antibody

1. Add 1 mL of 1× PBS to the second large antibody concentrator (C9) and close the cap.
2. Centrifuge at 3,000 × g for 6 minutes, ensuring that one membrane panel of the concentrator faces away from the center of the rotor.
3. Discard the flowthrough.
4. First add 2 mL of 1× PBS and then transfer the conjugated antibody solution to the large antibody concentrator.
5. Continue centrifugation in 3 minutes steps or until volume is reduced. Discard flowthrough.
6. Add 2 mL of fresh 1× PBS and centrifuge at 3,000 × g for 6 minutes, ensure retained volume is around 0.1 mL.
7. Recover the concentrated antibody by pipetting into a fresh 1.5 mL reaction tube.

   Ensure to recover the entire volume using smaller pipette tips!
8. *Optional: Wash the membrane of the large concentrator with 50 uL of 1× PBS.*
9. Determine the antibody concentration by measuring A280 (with A280 at 1.4 = 1 mg/mL) with a Nanodrop.

Troubleshooting

PROBLEM	POSSIBLE CAUSE	SOLUTION
Low conjugation efficiency or failed coupling (no/weak IF staining)	• Altered/insufficient Fc glycosylation of the antobody • The antibody solution is supplemented with carrier(s) • Presence of sodium azide • Weak azido modification • Incorrect antibody : MUSE activator ratio	• Use eukaryotically produced whole IgG with an intact Fc carbohydrate domain • The antibody should be BSA- and azide free. Azide can be removed during buffer exchange step • Follow the specified incubation condition: overnight at 37°C. • Recalculate the antibody concentration and reaction volume. Do not shorten the click incubation unless it is validated
Antibody loss during wash steps	• Breakage of concentrator • Partial retrieval of antibody concentrate from the concentrator	• Keep and measure concentration of the first flow-through with antibody • Make sure to recover all retained antibody solution; wash the membrane with fresh buffer if needed
Inconsistent results between conjugation runs	• The kit reagents, azido-modified antibody and antibody conjugate were frozen	• Avoid freeze-thaw and keep all reagents, intermediates and final conjugates at 4°C
High background in downstream assay	• Excess free MUSE® Activator left in antibody solution due to insufficient post-conjugation purification	• Perform the washing steps after the conjugation step to remove unconjugated MUSE activator

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 **ddH_2O**.
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 **ddH_2O** 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
No signal	• Sample/Tissue degradation • Poor antigen retrieval • MUSE® Reagents omitted • Different wash buffer than 2XSSCT used • Wrong mounting media • Wrong filter or imaging settings	• 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 2XSCCT • Use an antifade mountant compatible with the fluorophore(s) • Confirm microscope settings and channel assignment
Weak target staining	• 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	• 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 2XSCCT
Strong background staining	• 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)	• 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
Uneven staining - Edge effects	• Sample dried out during staining • Inconsistent reagent distribution	• Keep samples moist by covering after deparaffinization; use a humidified chamber

	• Inconsistent fixation of the sample • Inadequate permeabilization in thicker sections • Section thickness variability	• 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	• Spectral overlap of fluorophores due to panel imbalance • Antibody concentration to high • Amplification of highly abundant target • Imaging settings too broad •	• 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	• Prolonged light exposure during staining and /or imaging • High illumination intensity and repeated imaging of the same field • No antifade protection	• Minimize light exposure and reduce excitation intensity • Limit repeated scans • Mount samples in an antifade medium
Poor reproducibility	• Variable tissue, staining and imaging conditions	• 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.	• Stickiness of the amplifiers • Low signal of target	• Increase antibody concentration • Optimize microscope settings