Protocol

# *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 dd$H_2O$
   - 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 dd$H_2O$.
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<br>• The antibody solution is supplemented with carrier(s)<br>• Presence of sodium azide<br>• Weak azido modification<br>• Incorrect antibody : MUSE activator ratio | • Use eukaryotically produced whole IgG with an intact Fc carbohydrate domain<br>• The antibody should be BSA- and azide free. Azide can be removed during buffer exchange step<br>• Follow the specified incubation condition: overnight at 37°C.<br>• 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<br>• Partial retrieval of antibody concentrate from the concentrator | • Keep and measure concentration of the first flow-through with antibody<br>• 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 |