

Chemiluminescent Western Blot Kit

Cat#: orb342274 (ELISA Manual)

I. Overview

Biorbyt chemicals' Chemiluminescent Western Blot Kit for DYKDDDDK (FLAG®) combines all of the necessary reagents with a rapid proven protocol and the extremely high signal detection of our luminol chemiluminescent substrate for the detection of recombinant proteins containing the FLAG® epitope tag. The Chemiluminescent Western Blot Kit design includes straightforward procedures and color-coding to allow for ease of use. This kit contains sufficient substrate for up to 30 mini blots at 7.5 x 8 cm² (1,800 cm²) and is stable for at least 1 year when stored as indicated.

Please read the entire product insert prior to use.

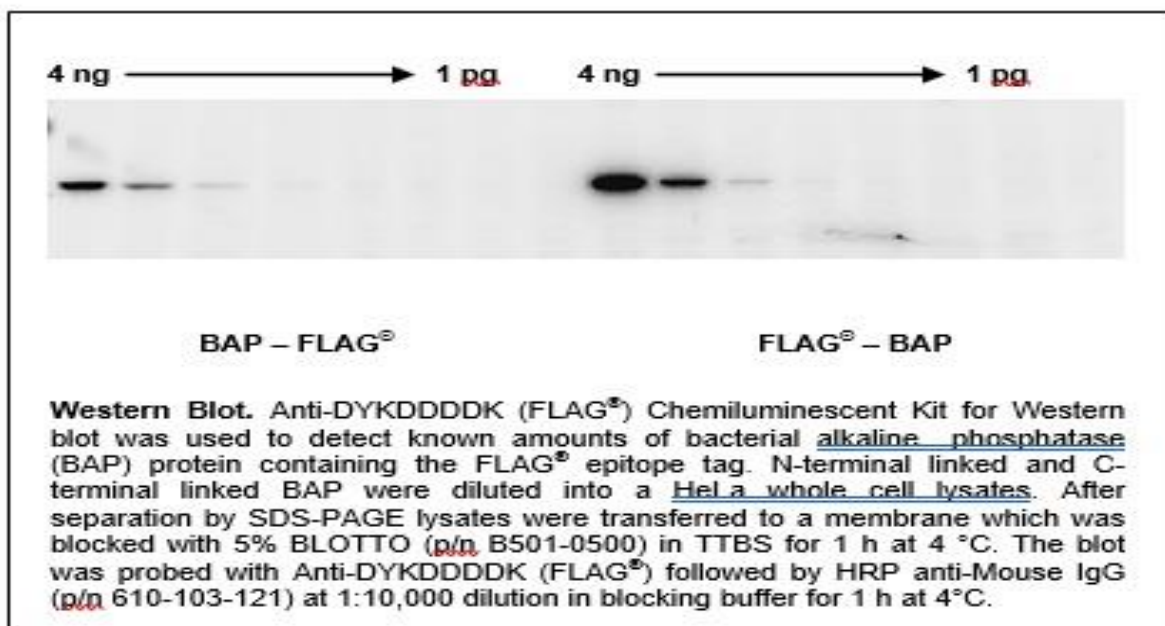
II. Kit Principle

This Chemiluminescent Western Blot Kit allows for the detection of FLAG® -tagged recombinant protein present in cell lysates provided by the user. After protein separation and transfer, the membrane is probed with Anti-DYKDDDDK (FLAG®). Detection of the membrane bound antibody-antigen complex is achieved by the addition of a secondary antibody conjugated to the enzyme horseradish peroxidase. The enzyme reacts with a specialized formulation of luminol, an extremely sensitive, non-radioactive substrate that emits light and allows visualization using X-ray film or other imaging methods, including highly sensitive CCD cameras and imaging systems.

The FLAG® epitope is a small but highly immunogenic peptide DYKDDDDK (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) which allows fusion proteins to retain their original conformation and function. The hydrophilic character of this sequence increases the likelihood that it will be located on the surface of FLAG® -tagged recombinant proteins for reaction with anti-DYKDDDDK (FLAG®) antibodies. The anti-DYKDDDDK (FLAG®) antibody included in this Western blot kit detects FLAG® -tagged recombinant proteins fused with FLAG® tag either at the amino-terminus or carboxy-terminus of the protein.

III. Intended Use

Use Biorbyt Immuno- chemicals' Anti-DYKDDDDK (FLAG®) Chemiluminescent Kit for Western Blotting for detection of FLAG® -tagged recombinant proteins by Western blot. This kit is useful for both "Western blotting" and "dot blotting" methods.



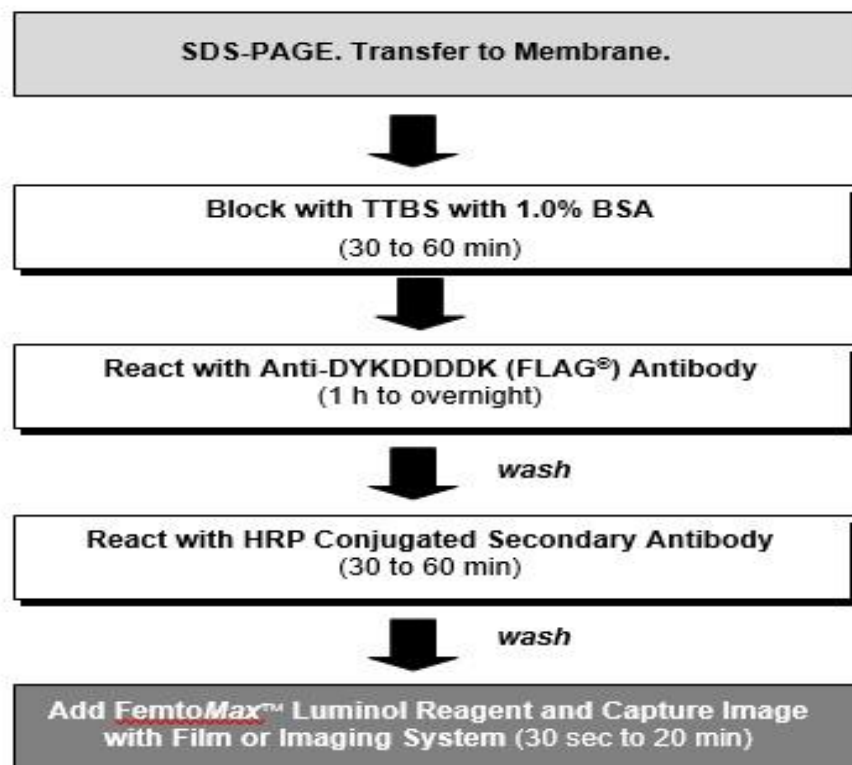
IV. Storage and Stability

This kit is stable for at least one year when stored as indicated upon receipt. Individual components are stable for 3-4 weeks after dilution when stored at +4°C. The FemtoMax[®] luminol chemiluminescent reagent is stable for up to 8 h at room temperature after mixing with buffer.

V. Number of Assays

Components in this kit are sufficient to run approximately 30 mini blots at 7.5 x 8 cm² (1,800 cm²). The amount of peroxidase conjugated secondary antibody supplied when diluted as recommended in our protocol will yield in excess of 200 mL of working solution. Adjustments in volumes for larger or smaller blots will affect the number of blots detected.

VI. Flow Diagram for Chemiluminescent Western Blot Procedure



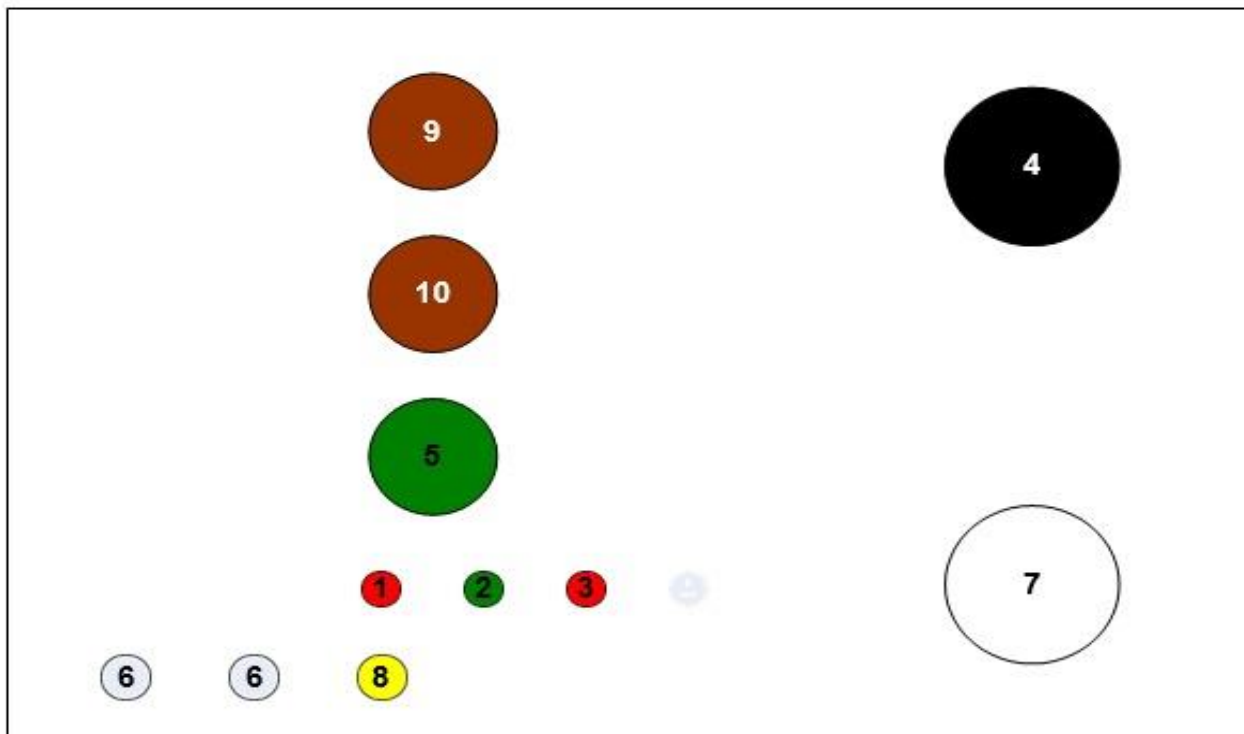
VII. Materials Required but Not Supplied

Nearly all components required for the detection of FLAG® -tagged recombinant protein by western blot are provided for your convenience in Biorbyt's Chemiluminescent Western Blot Kit for DYKDDDDK (FLAG®). Some additional materials not included are required:

- SDS-PAGE electrophoresis equipment and related materials
- Nitrocellulose, PVDF (polyvinylidene difluoride) or other membranes for protein transfer and transfer materials
- Microfuge tubes
- Rocker platform for gentle mixing during incubations
- X-ray film, cassettes and related materials or camera based imaging system
- Deionized water and molecular biology grade Tris base and sodium chloride

VIII. Kit Components and Layout

1. (1) x 100 μ L anti-DYKDDDDK (FLAG[®]) antibody in clear plastic 0.5 mL vial with **RED** cap. Store at -20° C.
2. (1) x 100 μ g Peroxidase anti-Mouse IgG antibody in amber plastic 0.5 mL vial with **GREEN** cap. Store at +4° C.
3. (1) x 100 μ g of control cell lysate containing FLAG[®]-tagged recombinant protein in clear plastic 0.5 mL vial with **RED** cap. Store at -20° C.
4. (1) x 50 mL Ultra Pure Tween-20 in an amber glass bottle with **BLACK** cap. Store at room temperature.
5. (1) empty dropper bottle with **GREEN** cap labeled "Diluted Secondary antibody".
6. (2) empty microfuge tubes for intermediate dilution of secondary antibody.
7. (1) x 10 g Bovine Serum Albumin (BSA) in a plastic bottle with a **WHITE** cap. Store at +4° C.
8. (1) x 0.5 mL Control Mouse IgG in plastic vial with **YELLOW** cap. Store at +4° C.
9. (1) x 10 mL FemtoMax Reagent A - Luminol - in an amber plastic bottle with **BROWN** cap. Store at +4° C.
10. (1) x 10 mL FemtoMax Reagent B - Reaction Buffer - in an amber plastic bottle with **BROWN** cap. Store at +4° C.
11. Incubation box with lid.
12. Instruction Manual.



IX. Solutions Required but Not Supplied

The user is to prepare the following buffers for this procedure. The exact volume of buffers required depends on the size of the membranes to be processed. We suggest preparation of 1.0 L of Tris Buffered Saline with Tween (TTBS) and 100 ml of TTBS with BSA. Prepare all solutions using ultra pure reagents and deionized (or equivalent) water. Filter the solutions and store at +4° C. Warm solutions to room temperature prior to use. Do not store solutions for more than one (1) month.

! **Note** - wash buffers **MUST NOT** contain SODIUM AZIDE or other inhibitors of peroxidase activity.

Buffer I Tris Buffered Saline with Tween-20 (TTBS)

Add 800 mL of deionized water. Dissolve 12.1 g of Tris base.

Dissolve 8.8 g Sodium Chloride (NaCl). Adjust pH to 7.5 with HCl.

Add 1.0 mL of Tween-20 (provided).

Adjust volume to 1.0 L with deionized water.

Buffer II TTBS with 1.0% (w/v) BSA

Add 100 mL of TTBS.

Dissolve 1.0 g of BSA (provided). Use solution immediately.

X. Preparation of Working Solutions

The Chemiluminescent Western Blot Kit for DYKDDDDK (FLAG®) comes with a concentrated stock of anti-DYKDDDDK (FLAG®) antibody (primary) and peroxidase conjugated anti-Mouse IgG antibody (secondary). Prior to use, dilute the primary antibody to a final concentration of 1µg/mL. Reconstitute and dilute the secondary antibody in a two-step process to a working solution. The working solution is ready for use. Reconstitute the peroxidase conjugated anti-Mouse IgG antibody by adding 100 µL of deionized water to the GREEN capped amber vial. Mix thoroughly and maintain this stock at 4 °C. Prepare an intermediate dilution (1:100) by pipetting 5 µL of the stock solution to 0.5 mL of Buffer I (TTBS) in a clean microfuge tube. Prepare a second dilution (1:200) by pipetting 50 µL of the intermediate solution to the GREEN capped dropper bottle labeled "Diluted Secondary Antibody." Add 10 mL of Buffer I (TTBS). Mix thoroughly. The final dilution will contain 50 ng/mL peroxidase conjugated goat anti- Mouse IgG antibody and will represent a 1:20,000 dilution of the stock solution.



- **FemtoMax[®] Luminol Substrate Reagent.** Just prior to use, prepare FemtoMax[®] Super Sensitive Chemiluminescent Substrate by mixing 1 mL of the Luminol chemiluminescent reagent (Reagent A) with 1 mL of the Reaction Buffer (Reagent B). Mix well. Protect from intense light. Keep working solution in an amber bottle. Normal laboratory light will not harm the working solution. Larger or smaller volumes of the substrate can be prepared by mixing components at the same 1:1 ratio.

XI. Western Blot Method

The following method is suggested as a guideline for the use of Biorbyt's Chemiluminescent Western Blot Kit for DYKDDDDK (FLAG®). Each researcher must optimize Western blotting conditions for their protein of interest. Membranes composed of nitrocellulose or PVDF can be used. Nylon membranes may also be used (see "Additional Notes"). After your antigen has been immobilized onto the membrane by transfer, dotting or filtration, follow the numbered steps below to process the blot. All reactions occur at room temperature. Use a rocking platform set at low speed for gentle agitation. Always add enough solution to cover the membrane. Never let a membrane air dry during this process. Add the suggested volumes or just enough volume to cover the membrane to keep it wet. Do not touch the membrane with your skin!

Note - some antigens in combination with the anti-DYKDDDDK (FLAG®) antibody may require specific conditions other than those stated below. If so, use these recommendations as a starting point for further optimization.

1. After transfer is complete, block the membrane by immersing in Buffer II (TTBS with BSA) and incubate at room temperature for 1 h with gentle agitation. The addition of 1.0% BSA (provided) as a blocking agent increases the signal-to-noise ratio when compared to using TTBS alone. Other blocking agents may be used (see "Additional Notes").
2. Aspirate or decant the blocking solution. Immediately add 5 ml of anti-DYKDDDDK (FLAG®) antibody (primary) solution diluted in Buffer I (TTBS) to the membrane. The appropriate dilution should be determined by the end user. We would recommend a starting dilution of 1:1,000. Greater dilutions often result in lower backgrounds but may require longer incubation times. Incubate for 1 h at room temperature with gentle agitation. If desired, the membrane can be incubated with anti-DYKDDDDK (FLAG®) antibody (primary) overnight at 4° C.
3. Aspirate or decant the anti-DYKDDDDK (FLAG®) antibody (primary) solution. Wash the blot with 3 changes of Buffer I for 5 min each with gentle agitation. Increasing the wash buffer volume or the number of washes may decrease background.
4. Aspirate or decant the wash solution and add 5 ml of the "Diluted Secondary Antibody" solution from the GREEN capped dropper bottle (see above for preparation). Incubate for 1 h at room temperature with gentle agitation.
5. Aspirate or decant secondary antibody solution. Wash the blot as in Step 3.
6. Prepare FemtoMax chemiluminescent reagent as described above just prior to use. Transfer blot to incubation box or film cassette then add 0.75 to 1.0 mL of freshly prepared FemtoMax reagent to the membrane (7.5 x 8.0 cm).
7. Immediately visualize the membrane by exposing X-ray film or by other imaging methods, including CCD camera based imaging systems. For film, expose and process the film according to the manufacturer's instructions. Cover the blot with clear plastic wrap or equivalent and remove any excess liquid and any air bubbles to reduce imaging artifacts. Start with a 60 sec exposure. Exposure times may be varied for best results. For imaging systems, follow the manufacturer's instructions and vary exposure times and/or binning for best results. The length of time required to achieve optimum signal varies greatly depending on several factors. Incubation times can range from 30 sec to 20 min or more.

XII. Additional Notes

- The methods given in these instructions are to be used as a guideline. Experienced users can make deviations from the stated method. Solutions have been optimized for the stated method and any change in reagent concentration, volume, reaction time or temperature will affect the overall performance of the kit. Generally, if a variable is to be modified, only alter one condition at a time.
- Nylon membrane is more difficult to block and may result in higher levels of non-specific staining. Using 10% BLOTTO (non-fat dry milk) is suggested to block nylon membranes. Allow the blocking step to proceed for several hours to overnight at 37° C. Do not use Tween-20 when using nylon membranes.
- The blocking of membranes for western blotting can be accomplished with TTBS only. We suggest adding BSA to lower non-specific staining. Users may omit BSA from the blocking step or use some other blocking agent, such as 5% normal goat serum, 3% fish gelatin, or other commercially available blocking agent (see p/n MB- 070), depending on previous experience.
- Always use enough solution to cover the membrane. Never let the membrane dry during the process.
- Protect the FemtoMax chemiluminescent reagent from light. Precise optimization is required to achieve maximum signal detection including optimizing the membrane, blocking conditions, antigen and antibodies. Detection by FemtoMax chemiluminescent reagent requires much less sample and antibody than most commercially available ECL substrates.
- Western blots can be repeatedly exposed to X-ray film to obtain optimal results or stripped of detection reagents and re-probed.
- Use the same blotting conditions for FemtoMax chemiluminescent reagent as you would for Amersham ECL Plus™ Substrate or Pierce SuperSignal West Femto Substrate.
- Use care not to touch the membrane with your skin! Wear gloves. Make certain that all equipment used in the process is free of foreign material.
- All reactions occur at room temperature.
- Use a rocking platform set at low speed for gentle agitation for all incubation steps.
- Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used to dilute the secondary antibody, substrate or any other FemtoMax chemiluminescent reagent.
- A positive control mouse IgG is provided in a YELLOW capped vial for use as a secondary antibody control. Spot 1 or 2 μ L as a control on your Western or dot blot prior to the blocking step.
- Store the components of this kit at as specified.
- Individual components of this kit may be ordered separately (see "Replacement Parts List").

XIII. Troubleshooting Guide Little or no signal

Incomplete transfer of proteins. Follow all protocols included with your transfer apparatus. Check for the presence of transferred proteins using India ink stain as described in Reference 1. Poor binding of anti-DYKDDDDK (FLAG®) antibody (primary). Use provided control cell lysate containing FLAG® -tagged recombinant protein in serial dilution as a control. Decrease the dilution (increase the concentration) of anti-DYKDDDDK (FLAG®) antibody. Increase the incubation time of anti-DYKDDDDK (FLAG®) antibody from 30 minutes to several hours or overnight. Increase the incubation temperature to 37° C.

Poor binding of peroxidase conjugated anti-IgG. Include 1 or 2 μ L of Mouse IgG as a control in your western blot or dot blot to ensure that the FemtoMax kit components are performing as described.

Inactive Peroxidase Conjugate. Be certain that all buffers are free of sodium azide, which is a strong inhibitor of peroxidase activity.

Multiple signals

Too much protein on the blot. Verify the concentration of your protein sample, using Bradford or BCA reagent. For best results, load approximately 10 µg of total protein (lysate) per lane.

Too high concentration of anti-DYKDDDDK (FLAG®) antibody. Increase the dilution of anti-DYKDDDDK (FLAG®) antibody solution.

Overexposure of signal. Decrease exposure time of film or decrease settings on camera system to decrease the signal from minor bands.

High background / Poor signal-to-noise ratio

Insufficient blocking. Be certain blocking buffer has been properly prepared. In most cases, the addition of 1.0% BSA will decrease background over the use of TTBS alone. In some cases, increased concentrations of BSA (up to 5%) are necessary.

Insufficient Washing. Increase the number of wash steps and the volume of TTBS used for each wash.

XIV. References

Antibodies, A Laboratory Manual. Ed Harlow and David Lane, eds. Cold Spring Harbor Press. 1988. Chapter 12 gives an excellent overview of Western Blotting techniques, including India Ink staining.

Current protocols in Molecular Biology. J. Ausubel, et al, eds. John Wiley and Sons, New York. Gives a complete protocol of Western Blotting and Dot Blotting.

Molecular Cloning: A Laboratory Manual. 2nd Edition. J. Sambrook, E.F. Fritsch and T. Maniatis, eds. Cold Spring Harbor Press, 1989. Chapter 18 gives detailed protocols for both the production of cell lysates and electrophoresis and blotting of proteins.

Antibodies, A Practical Approach. 2nd Edition. Catty, D., ed. IRL Press, Oxford, England. 1990. Volumes I and II represent a detailed and complete reference for most current antibody techniques.

XV. Trademarks

This material is subject to proprietary rights and is sold under license from Biorbyt. This product is licensed for sale only for 'research-use' only. There is no implied license hereunder for any commercial use. FemtoMax is a trademark of Biorbyt. Anti-FLAG® and FLAG® are registered trademarks of Sigma-Aldrich Biotechnology LP and Sigma-Aldrich Co.

XVI. Disclaimer

This product is for research use only and is not intended for therapeutic or diagnostic applications. Please contact a technical service representative for more information. All products of animal origin manufactured by Biorbyt'ss are derived from starting materials of North American origin. Collection was performed in

United States Department of Agriculture (USDA) inspected facilities and all materials have been inspected and certified to be free of disease and suitable for exportation. All properties listed are typical characteristics and are not specifications. All suggestions and data are offered in good faith but without guarantee as conditions and methods of use of our products are beyond our control. All claims must be made within 30 days following the date of delivery. The prospective user must determine the suitability of our materials before adopting them on a commercial scale. Suggested uses of our products are not recommendations to use our products in violation of any patent or as a license under any patent of Biobyte, Inc.

XVII. Additional Products and Services

Additional Chemiluminescent Western Blotting Kits

Product	Code	Size	Price
FemtoMax ™ Chemiluminescent Western Blot Kit for use with Human Primary Antibody	KCA001	1 each	inquire
FemtoMax ™ Chemiluminescent Western Blot Kit for use with Mouse Primary Antibody	KCA002	1 each	inquire
FemtoMax ™ Chemiluminescent Western Blot Kit for use with Rabbit Primary Antibody	KCA003	1 each	inquire
FemtoMax ™ Chemiluminescent Western Blot Kit for use with Goat Primary Antibody	KCA004	1 each	inquire

Replacement Parts for DYKDDDDK (FLAG®) Chemiluminescent Kit for Western Blotting

Anti-DYKDDDDK (FLAG®) Mouse MAb antibody	200-301-383	100 µg	inquire	BSA, Protease and IgG Free	BSA-10	10 g	inquire
Anti-DYKDDDDK (FLAG®) Rabbit PAb antibody	600-401-383	250 µg	inquire	<u>Ultra Pure</u> Tween-20	TW0020	50 mL	inquire
Peroxidase Anti-Mouse IgG	610-1319	2 mg	inquire	10X TTBS pH 7.5	MB-013	1.0 L	inquire
Anti-DYKDDDDK (FLAG®) antibody Agarose Conjugate	200-350-383	1 cc	inquire	BLOTTO Immunoanalytical Grade (Non-Fat Dry Milk)	B501-0500	500 g	inquire
FemtoMax ™ Super Sensitive Chemiluminescent Substrate	FEMTOMAX-110	110 mL	inquire	Western Incubation Box SMALL	WIB-2875-010	10 PACK	inquire
Control Mouse IgG	KCC002	0.5 mL	inquire	Western Incubation Box LARGE	WIB-4625-005	5 PACK	inquire