

Human TNFSF11/RANKL AccuSignal™ ELISA Kit

AccuSignal high sensitivity sandwich ELISA (Enzyme-Linked Immunosorbent Assay) Kit is *an in vitro* enzyme-linked immunosorbent assay designed for the quantitative detection of Human TNFSF11/RANKL in cell culture supernatants, serum and plasma (heparin, EDTA).

KIT COMPONENTS

| Human TNFSF11/RANKL AccuSignal™ ELISA Kit | | | |
|---|----------------|---|-------------------------|
| Component | Catalog Number | Description | Size |
| Antibody Coated ELISA Plate | KOA0505A | 96-well plate pre-coated with anti-Human TNFSF11/RANKL antibody | 1 |
| Target Protein Standard | KOA0505B | lyophilized recombinant Human TNFSF11/RANKL | 2 vials (10 ng/vial) |
| Biotinylated Detection Antibody | KOA0505C | biotinylated anti-Human TNFSF11/RANKL antibody | 130 µl (dilution 1:100) |
| ABC Complex | KAB0101 | Avidin-Biotin-Peroxidase Complex (ABC) | 130 µl (dilution 1:100) |
| Sample Diluent | KAE0101 | sample diluent buffer | 30 ml |
| Antibody Diluent | KAF0101 | antibody diluent buffer | 12 ml |
| ABC Diluent | KAG0101 | ABC diluent buffer | 12 ml |
| Substrate Solution | KAC0101 | TMB color developing reagent | 10 ml |
| Stop Solution | KAD0101 | TMB stop solution | 10 ml |
| Adhesive Cover | - | Adhesive cover | 4 |

I. STORAGE CONDITIONS

Store at 4°C for 6 months, or at -20°C for 12 months. Avoid multiple freeze-thaw cycles.

II. INTRODUCTION

Human TNFSF11/RANKL ELISA Kit is based on standard sandwich enzyme-linked immune-sorbent assay technology. This assay employs a monoclonal antibody specific for TNFSF11/RANKL pre-coated onto 96-well plate. Standards (Expression system for standard: NSO; Immunogen sequence: G64-D245) and test samples are pipetted into the wells, and a biotinylated detection polyclonal antibody from goat specific for TNFSF11/RANKL is added to the wells. The wells are washed with PBS or TBS buffer. After washing away unbound biotinylated antibody, Avidin-Biotin-Peroxidase Complex is added to the wells and unbound conjugates are washed away with PBS or TBS buffer. HRP substrate TMB solution is added to the wells to visualize HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product. The addition of stop solution changes the color from blue to yellow. The density of yellow is proportional to the amount of Human TNFSF11/RANKL amount captured in plate.

III. REQUIRED EQUIPMENT AND REAGENTS

- Microplate reader in standard size
- Automated plate washer
- Adjustable pipettes and pipette tips
- Clean tubes and Eppendorf tubes
- Washing buffer (neutral PBS or TBS)
 - Preparation of 0.01M TBS:
Add 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 1000 ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.
 - Preparation of 0.01 M PBS:
Add 8.5 g sodium chloride, 1.4 g Na₂HPO₄ and 0.2 g NaH₂PO₄ to 1000 ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

IV. GENERAL CONSIDERATIONS

Please read the following instructions before starting the experiment.

- To inspect the validity of experimental operation and the appropriateness of sample dilution proportions, pilot experiment using standards and a small number of samples is recommended.
- The TMB Color Developing agent is colorless and transparent before using, contact us if it is not the case.
- Before using the Kit, briefly spin down the vials.
- For statistical reasons, we recommend both standard and samples should be assayed with a minimum of two replicates (duplicates).
- Do not let 96-well plate to dry, this will inactivate active components on plate.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Do not mix or substitute reagents or materials from other kit lots or vendors.
- In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution be pre-warmed in 37°C for 30 min before using.

V. SANDWICH ELISA PROTOCOL

A. Sample Preparation

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

- **Serum:**
Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature.
Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store at -20°C.

- **Cell culture supernatants:**
Remove particulates by centrifugation, assay immediately or aliquot and store at -20°C.
- **Plasma:**
Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 min at 1500 x g within 30 min of collection. Assay immediately or aliquot and store samples at -20°C.

B. Sample Dilution

Please note that levels of the target protein may vary between different samples. Estimation of the concentration of a target protein in the sample and the optimal dilution factors for each sample must be determined by the investigator. The diluted target protein concentration should fall near the middle of the linear regime in the standard curve. Sample diluent buffer should be used for dilution of samples.

Following are the suggested guidelines for sample dilution. **The samples must be mixed well with the diluent buffer.**

- **High target protein concentration (50000pg/ml-500000pg/ml)**
The working dilution should be 1:100. For example, add 1µl sample into a tube with 99µl sample diluent buffer.
- **Medium target protein concentration (5000pg/ml-50000pg/ml)**
The working dilution should be 1:10. For example, add 10µl sample into a tube with 90µl sample diluent buffer.
- **Low target protein concentration (78pg/ml-5000pg/ml)**
The working dilution should be 1:2. For example, add 50µl sample into a tube with 50µl sample diluent buffer.
- **Very Low target protein concentration (0pg/ml-78pg/ml)**
No dilution necessary, or the working dilution should be 1:2.

C. Reagent Preparation

Reconstitution of the Human TNFSF11/RANKL standard:

Human TNFSF11/RANKL standard solution should be prepared no more than 2 hours prior to the experiment. Two vials of TNFSF11/RANKL standard (10ng/vial) are provided with each kit. Use one vial/experiment.

- For preparation of 10,000pg/ml of Human TNFSF11/RANKL standard solution, add 1ml sample diluent buffer into one vial, keep the vial at room temperature for 10 min and mix thoroughly.
- For preparation of 1000pg/ml of standard solution, add 0.1ml of the above Human TNFSF11/RANKL standard solution into 0.9ml of sample diluent buffer and mix thoroughly.
- For preparation of 500pg/ml to 15.625pg/ml of Human TNFSF11/RANKL standard solutions, label 6 Eppendorf tubes with 500pg/ml (tube#1), 250pg/ml (tube#2), 125pg/ml (tube#3), 62.5pg/ml (tube#4), 31.25pg/ml (tube#5), and 15.625pg/ml (tube#6) respectively.
- Pipette 300µl of the sample diluent buffer into each tube. Add 300µl of the above 1000pg/ml TNFSF11/RANKL standard solution into tube #1 and mix thoroughly.
- Transfer 300µl from tube #1 to tube #2 and mix thoroughly.
- Transfer 300µl from tube #2 to tube #3 and mix thoroughly, and prepare further serial dilutions.

Note: The standard solutions are best used within 2 hours. The 10,000pg/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

Preparation of biotinylated anti-Human TNFSF11/RANKL antibody working solution:

The solution should be prepared no more than 2 hours prior to the experiment.

- The total volume should be 0.1ml/well x (the number of wells). Prepare 100-200µl more of the solution than the total volume required to compensate for pipetting errors.
- Biotinylated anti-Human TNFSF11/RANKL antibody should be diluted 1:100 with the antibody diluent buffer and mixed thoroughly. For example, add 1µl Biotinylated Anti-Human TNFSF11/RANKL antibody to 99µl antibody diluent buffer.

Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution:

The solution should be prepared no more than 1 hour prior to the experiment.

- The total volume should be: 0.1 ml/well x (the number of wells). Prepare 0.1-0.2 ml more of the solution than total volume required.
- Avidin-Biotin-Peroxidase Complex (ABC) should be diluted 1:100 with the ABC dilution buffer and mixed thoroughly. For example, add 1µl ABC to 99µl ABC diluent buffer.

D. Assay Procedure

ABC working solution and TMB substrate reagent should be warmed at 37°C for 30 min prior to use. Samples and reagents should be mixed thoroughly and evenly after dilution. A standard curve for TNFSF11/RANKL detection should be prepared for each experiment. It is recommended to determine sample fold dilution by simple estimation of TNFSF11/RANKL amount in the samples.

1. Aliquot 100µl per well of each Human TNFSF11/RANKL standard (1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.25pg/ml, and 15.625pg/ml) into pre-coated 96-well plate. Add 100µl of the sample diluent buffer into control wells. Add 100µl of diluted sample (Human cell culture supernatants, serum, plasma {heparin} into appropriate wells). See "Sample Dilution Guideline" above for details. **It is recommended to assay all standards, controls and samples in duplicate.**
2. Seal the plate with provided adhesive plastic cover and incubate at 37°C for 90 min.
3. Remove the cover and discard the solution. Invert the plate and blot it against clean paper towels or other absorbent material. Do NOT let the wells dry completely at any time.
4. Add 100µl of biotinylated anti-Human TNFSF11/RANKL antibody working solution to each well, seal the plate with adhesive plastic cover and incubate at 37°C for 60 min.
5. Discard the solution and wash the plate 3 times with 0.01M TBS or 0.01M PBS (wash buffer) and each time let washing buffer stay in the wells for ~1 min. Wash by filling each well with 300µl of wash buffer using a multi-channel pipette or auto washer. Complete removal of liquid at each step is essential to good performance. (Note: for automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling the wells with PBS or TBS buffer). After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels or other absorbent material.
6. Add 100µl of prepared ABC working solution to each well, seal the plate and incubate at 37°C for 30 min.
7. Wash plate 5 times with 0.01M TBS or 0.01M PBS as described in step 5.
8. Add 90µl of TMB substrate reagent to each well, seal the plate and incubate for 25-30 min at 37°C in the dark. (Note: the optimal incubation time should be determined by the end user).
9. Add 100µl of stop solution to each well. The color will change to yellow immediately.

10. Read at 450 nm in a microplate reader within 30 min after adding the stop solution.

Calculations

For calculations, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The Human TNFSF11/RANKL concentration of the samples can be interpolated from the standard curve.

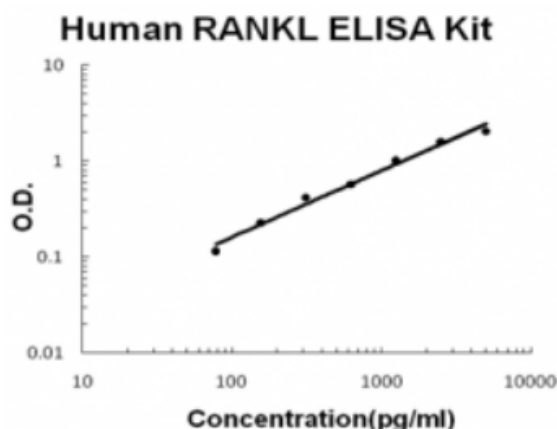
Note: if the samples measured were diluted, multiply the concentrations obtained from interpolation by the dilution factor.

VI. DATA OBTAINED FROM Human TNFSF11/RANKL

| Concentration (pg/ml) | 0 | 78 | 156 | 313 | 625 | 1250 | 2500 | 5000 |
|-----------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| O.D | 0.017 | 0.115 | 0.227 | 0.416 | 0.573 | 1.015 | 1.591 | 2.039 |

(TMB reaction incubation at 37°C for 25-30min)

VII. Human TNFSF11/RANKL ACCUSIGNAL™ ELISA KIT STANDARD CURVE



This standard curve was generated for demonstration purpose only (A standard curve must be run with each assay).

| | |
|-------------------------|---|
| Range | 78pg/ml-5000pg/ml |
| Sensitivity | <10pg/ml |
| Specificity | Natural and recombinant Human TNFSF11/RANKL |
| Cross-reactivity | There is no detectable cross-reactivity with other relevant proteins. |

INTRA AND INTER ASSAY PRECISION

Intra-Assay Precision (Precision within an assay): Three samples of known concentration were tested on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays): Three samples of known concentration were tested in separate assays to assess inter-assay precision.

| Sample | Intra-Assay Precision | | | Inter-Assay Precision | | |
|---------------------------|-----------------------|------|-------|-----------------------|-------|-------|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| N | 16 | 16 | 16 | 24 | 24 | 24 |
| Mean(pg/ml) | 624 | 1302 | 2684 | 714 | 1428 | 2710 |
| Standard deviation | 20.6 | 66.4 | 123.5 | 28.56 | 92.82 | 154.5 |
| CV (%) | 3.3 | 5.1 | 4.6 | 4 | 6.5 | 5.7 |

VIII. ASSAY SUMMARY

- Prepare all reagents, samples and standards as instructed.
- Add standards or samples to each well used. Incubate the plate at 37°C for 90 min. Do not wash.
- Add prepared biotinylated antibody to each well. Incubate the plate at 37°C for 60 min. Wash plate 3 times with 0.01M TBS or PBS.
- Add prepared ABC working solution. Incubate the plate at 37°C for 30 min. Wash plate 5 times with 0.01M TBS or TBS.
- Add TMB developing reagent to each well. Incubate the plate at 37°C in dark for 25-30 min.
- Add TMB stop solution. Read at 450nm.

IX. TROUBLESHOOTING

Weak or No Signal

| | Possible Cause | Solution |
|---|--------------------------------------|--|
| 1 | Problem with the standard | Use new sample Check that the standard is appropriately handled |
| 2 | Incubation time too short | Follow the exact guidelines for incubation time (If the problem persists, try incubating samples at 4°C overnight) |
| 3 | Incubation temperature too low | Ensure incubations are done at correct temperature Before proceeding |
| 4 | Incompatible sample type | Use sample that the assay is known to detect as a positive control (Include such control in your experiment) |
| 5 | Incompatible assay buffer | Ensure assay buffer is compatible with the target of interest |
| 6 | Target present below detection limit | Decrease dilution factor or concentrate samples |
| 7 | Incorrect/Insufficient/No substrate | Check the substrate identity Increase concentration or amount of substrate |

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| 8 | Antibody stored at 4°C for several weeks or subjected to repeat freeze-thaw cycles | Use fresh aliquot of antibody that has been stored at -20°C or below |
| 9 | Incorrect reagents added/prepared; Missing reagents | Check protocol, ensure correct reagents are added in proper order and prepared to correct concentrations |
| 10 | Expired/Contaminated reagents | Prepare fresh/uncontaminated reagents |
| 11 | Enzyme inhibitor present | Avoid sodium azide in HRP reactions |
| 12 | Incorrect storage of components | Check storage conditions for the kit (Kit need to be stored at 4°C) |
| 13 | Excessive plate washing | Gently pipette wash buffer (manual method) Ensure correct pressure (automatic wash system) |
| 14 | Wells dry out | Cover plate using adhesive cover at all incubation times |
| 15 | Plate read at incorrect detection wavelength | Use recommended wavelength/filter Ensure plate reader is set correctly for substrate used |
| 16 | Slow color development | Prepare substrate immediately before use Allow longer incubation time Ensure stock solution is unexpired and uncontaminated |

Saturated Signal

| | Possible Cause | Solution |
|---|--|--|
| 1 | High sample concentration | Use higher sample dilutions (Determine the optimal dilutions by titration assay) |
| 2 | Excessive substrate | Decrease concentration or amount of substrate: The substrate provided with the ELISA kit might require further dilution |
| 3 | Substrate color changed before use | Prepare substrate immediately before use |
| 4 | Non-specific antibody binding | Use affinity-purified antibody and preferably one that is pre-adsorbed. |
| 5 | Incubation time too long | Follow the exact guidelines (If the problem persists, try incubating samples at 4°C overnight) |
| 6 | Excessive antibody | Repeat the assay with lower antibody concentrations to find the optimal one for your experiment |
| 7 | Contaminated buffers or HRP | Prepare and use fresh buffers |
| 8 | Insufficient washing | Follow the exact guidelines At the end of each washing step, flick the plate over a sink and dry the plate on a paper towel |
| 9 | Plate adhesive cover not used or re-used | During incubations, cover plates with adhesive cover. Use a fresh cover every time the used cover is removed from the plate |

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|----|------------------------------------|---|
| 10 | Plate read at incorrect wavelength | Use recommended wavelength/filter Ensure plate reader is set correctly for the substrate used |
| 11 | Excess time before plate reading | Read your plate within 30 minutes after adding the substrate (If the reading is not performed within this time frame, add a stopping solution after sufficient color is developed in the plate) |

High Background

| | Possible Cause | Solution |
|----|---|--|
| 1 | Insufficient washing | Follow the exact guidelines At the end of each washing step, flick the plate over a sink and pat dry the plate on a paper towel |
| 2 | Excessive antibody | Repeat the assay with lower antibody concentrations to find the optimal one for your experiment |
| 3 | Excessive substrate | Decrease concentration or amount of substrate |
| 4 | Cross reactivity | Run appropriate controls |
| 5 | Non-specific antibody binding | Use affinity-purified antibody and preferably one that is pre-adsorbed |
| 6 | Insufficient Tween in buffers | Use PBS or TBS containing 0.05% Tween |
| 7 | Suboptimal salt concentration in washing buffer | Optimize salt concentration as high concentration can reduce non-specific interactions |
| 8 | Incubation temperature too high | Optimize incubation temperature for your assay (antibodies bind optimally at very specific temperature) |
| 9 | Reagents were not mixed properly | Thoroughly mix all reagents and samples before pipetting solutions into wells |
| 10 | Blanks contaminated with samples | Change pipette tips when switching between blanks and samples |
| 11 | Sample contaminated with enzymes | Test samples with substrate alone to check for contaminating enzymes |
| 12 | Contaminated TMB substrate | Use a clean container to check that the substrate is not contaminated (TMB substrate should be clear and colorless before adding to wells) |
| 13 | Substrate exposed to light | Carry out substrate incubation in dark |

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| 14 | Evaporation of solution from well during incubation | Always incubate with a cover on the plate |
| 15 | Incubation time too long | Follow the exact guidelines for incubation times (If the problem persists, try incubating samples at 4°C overnight) |
| 16 | Incorrect standard curve dilutions | Check pipetting techniques Check calculations |
| 17 | Unstopped color development | Use Stopping solution to prevent over-development |
| 18 | Excessive time lapsed before plate reading | Read your plate within 30 minutes after adding the substrate (If the reading is not performed within this time frame, add a stopping solution after sufficient color is developed in the plate) |
| 19 | Incorrect plate reading setting | Use recommended wavelength/filter Ensure plate reader is set correctly for the substrate used |

Low Sensitivity

| | Possible Cause | Solution |
|---|---|--|
| 1 | Improper storage of ELISA kit | Store all reagents as recommended |
| 2 | Insufficient target | Reduce sample dilution or concentrate sample |
| 3 | Inactive substrate | Ensure reporter enzyme has the expected activity |
| 4 | Insufficient substrate | Increase concentration or amount of substrate |
| 5 | Incompatible sample type | Include positive control in your experiment |
| 6 | Interfering ingredients in buffers and sample | Check reagents for any interfering chemicals, e.g. sodium azide in antibodies inhibit HRP enzyme; EDTA used as anti-coagulant for plasma collection inhibits enzymatic reactions |
| 7 | Mixing or substituting reagents from different kits | Avoid mixing components from different kits |
| 8 | Incorrect plate reading setting | Use recommended wavelength/filter Ensure plate reader is set correctly for the substrate used |

Poor Standard Curve Generation

| | Possible Cause | Solution |
|---|-----------------------------------|--|
| 1 | Improper standard solution | Confirm dilutions are done correctly Prepare new standard curve as appropriate |
| 2 | Standard improperly reconstituted | Briefly spin vial before opening Inspect for undissolved material after reconstituting |
| 3 | Standard degraded | Store and handle standard as recommended Prepare standards no more than two hours before use |
| 4 | Pipetting error | Use calibrated pipettes and proper pipetting technique |
| 5 | Insufficient washing | Follow the exact guidelines At the end of each washing step, flick the plate over a sink and pat dry the plate on a paper towel |
| 6 | Poorly mixed reagents | Thoroughly mix reagents |
| 7 | Plates stacked during incubation | Keep plates separated if not using rotating plates |

Poor Replicate Data

| | Possible Cause | Solution |
|---|--|---|
| 1 | Bubbles in wells | Ensure no bubbles are present prior to reading the plate |
| 2 | Insufficient washing of wells | Carefully wash wells Check that all ports of the plate washer are unobstructed |
| 3 | Incomplete reagent mixing | Ensure all reagents are mixed thoroughly |
| 4 | Inconsistent pipetting | Use calibrated pipettes and proper pipetting techniques Use a new cover every time the used cover is removed from the plate |
| 5 | Inconsistent sample preparation or storage | Ensure consistent sample preparation and optimal sample storage (e.g. minimize freeze/thaw cycles) |
| 6 | Particulates in samples | Remove the particulates by centrifugation |
| 7 | Cross-well contamination | Ensure plate covers and pipette tips are not contaminated with reagents |
| 8 | Edge effect (higher or lower OD in peripheral wells than in central wells) | Ensure plates and reagents are kept at temperatures as instructed During incubation, seal the plate completely and avoid stacking plates |

Inconsistent Assay-to-Assay Results

| | Possible Cause | Solution |
|---|--------------------------------|---|
| 1 | Insufficient washing of wells | Carefully wash wells Check that all ports of the plate washer are unobstructed |
| 2 | Varied incubation temperatures | Adhere to recommended incubation temperature |

| | | |
|---|---------------------------------|--|
| 3 | Variation in protocol | Adhere to the same protocol from experiment to experiment |
| 4 | Plate cover not used or re-used | During incubations, cover plates with plate cover Use a new cover every time the used one is removed |
| 5 | Incorrect dilutions | Confirm dilutions are done correctly for standard solutions Prepare new standard curve as appropriate |
| 6 | Contaminated buffers | Prepare and use fresh buffers |

Slow Color Development

| | Possible Cause | Solution |
|---|--|---|
| 1 | Substrates too old, contaminated or used at incorrect pH | Prepare fresh substrates at correct pH |
| 2 | Expired/Contaminated solutions | Prepare fresh reagents before use |
| 3 | Incorrect incubation temperature | Ensure plates and reagents are kept at temperatures as instructed During incubation, seal the plate completely and avoid stacking plates |
| 4 | Low antibody concentration | Repeat the assay with higher antibody concentrations to find the optimal one for your experiment |

Plate Imaging Problem

| | Possible Cause | Solution |
|---|--|--|
| 1 | Oversaturated image after acquisition | Use full resolution image to analyze results (Do not use jpeg or other compressed formats) |
| 2 | Blurry spots in images | Re-focus your camera before taking a new image |
| 3 | Repeated pixel values or rectangular spots | Use lower bin size, higher image resolution and/or lossless file type |
| 4 | Flat standard in images | Reduce acquisition time |

X. RELATED PRODUCTS

| Component | Catalog # | Size |
|-----------------|------------------------|---------|
| 10x TBS pH 7.5 | MB-012 | 1000 ml |
| 10x TTBS pH 7.5 | MB-013 | 1000 ml |

| | | |
|-----------------|-----------------------------|---------|
| 10X PBS pH 7.2 | MB-008 | 1000 ml |
| 10x PBST pH 7.2 | MB-075-1000 | 1000 ml |