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# ab199083 – Lipocalin-2 (NGAL) Mouse SimpleStep ELISA® Kit

Instructions for Use

For the quantitative measurement of Lipocalin-2 (NGAL) in mouse serum, plasma, urine, and cell culture supernatant.

This product is for research use only and is not intended for diagnostic use.

Last Updated 18 January 2019

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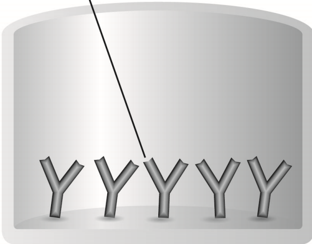
## 1. BACKGROUND

Lipocalin-2 (NGAL) mouse *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Lipocalin-2 protein in mouse serum, plasma, urine and cell culture supernatant.

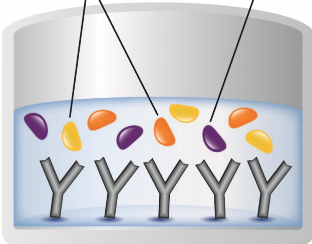
The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Lipocalin-2 (also known as Neutrophil gelatinase-associated lipocalin or NGAL) is an iron binding and iron trafficking protein. Lipocalin-2 is involved in multiple cellular processes including apoptosis, innate immunity and renal development. Mice deficient in Lipocalin-2 appear normal but have increased susceptibility to bacterial infection. The bacteriostatic function may be related to Lipocalin-2 limiting bacterial iron supply. Mouse Lipocalin-2 has 81% and 62% protein sequence identity to rat and human Lipocalin-2, respectively.

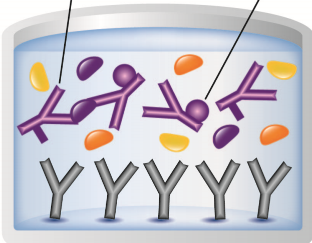
## 2. ASSAY SUMMARY



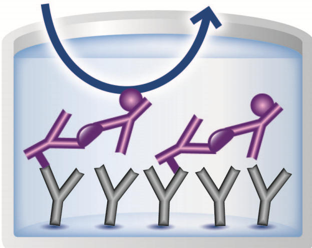

Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.




Add standard or sample to appropriate wells.




Add Antibody Cocktail to all wells. Incubate at room temperature.




Aspirate and wash each well. Add TMB Development Solution to each well and incubate. Add Stop Solution at a defined endpoint.

Alternatively, record color development kinetically after TMB substrate addition.

## 3. PRECAUTIONS

**Please read these instructions carefully prior to beginning the assay.**

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

## 4. STORAGE AND STABILITY

**Store kit at 2-8ºC immediately upon receipt.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Reagent and Standard Preparation sections.

## 5. MATERIALS SUPPLIED

| Item | Amount | Storage Condition (Before Preparation) |
|---|---|---|
| 10X Mouse Lipocalin-2 Capture Antibody | 600 µL | +2-8ºC |
| 10X Mouse Lipocalin-2 Detector Antibody | 600 µL | +2-8ºC |
| Mouse Lipocalin-2 Lyophilized Recombinant Protein | 2 Vials | +2-8ºC |
| Antibody Diluent 5B | 6 mL | +2-8ºC |
| 10X Wash Buffer PT | 20 mL | +2-8ºC |
| TMB Development Solution | 12 mL | +2-8ºC |
| Stop Solution | 12 mL | +2-8ºC |
| Sample Diluent NS | 50 mL | +2-8ºC |
| Pre-Coated 96 Well Microplate (12 x 8 well strips) | 96 Wells | +2-8ºC |
| Plate Seal | 1 | +2-8ºC |

## 6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

## 7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

## 8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- The provided Antibody Diluents and Sample Diluents contain protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

## 9. REAGENT PREPARATION

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. **The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.**
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

### 9.1 1X Wash Buffer PT

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.

### 9.2 Antibody Cocktail

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 5B. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 5B. Mix thoroughly and gently.

## 9.3 STANDARD PREPARATION

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.

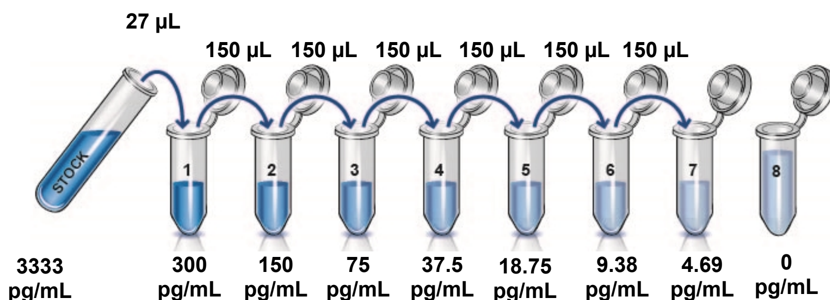
The following table describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 Reconstitute the Lipocalin-2 mouse lyophilized recombinant protein standard sample by adding 1 mL Sample Diluent NS by pipette. Mix thoroughly and gently. Hold at room temperature for 10 minutes and mix gently. This is the 3333 pg/mL **Stock Standard** Solution.

10.2 Label eight tubes, Standards 1– 8.

10.3 Add 273 µL Sample Diluent NS into tube number 1 and 150 µL of Sample Diluent NS into numbers 2-8.

10.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



## 10. SAMPLE PREPARATION

| TYPICAL SAMPLE DYNAMIC RANGE | |
|---|---|
| **Sample Type** | **Range (%)** |
| Mouse Serum | 0.001-0.05 |
| Mouse Plasma - Citrate | 0.001-0.05 |
| Mouse Plasma (platelet poor EDTA) | 0.001-0.05 |
| Mouse Urine | 0.1-1 |
| Stimulated L929 Supernatant | 0.3-10 |
| Stimulated RAW 264.7 Supernatant | 0.01-0.1 |
| Stimulated J774A.1 Supernatant | 0.1-5 |

11.1 **Plasma**

Collect plasma using citrate or EDTA. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples into Sample Diluent NS and assay. Store un-diluted plasma samples at -20ºC or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 **Serum**

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples into Sample Diluent NS and assay. Store un-diluted serum at -20ºC or below. Avoid repeated freeze-thaw cycles.

11.3 **Cell Culture Supernatants**

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.4 **Urine**

Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Collect supernatants, dilute in Sample Diluent NS and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

*Note*: Due to the high dilutions required for certain mouse samples, we recommend initially diluting your samples in 1X Wash Buffer and then performing the final dilution in Sample Diluent NS. As an example the table below demonstrates this:

| Tube # | Sample to Dilute | Volume of Sample (µL) | Volume of 1X Wash Buffer (µL) | Volume of Sample Diluent NS (µL) | Starting Conc. | Final Conc. |
|---|---|---|---|---|---|---|
| 1 | Neat serum/ plasma | 4 | 196 | - | Neat | 2% |
| 2 | Tube #1 | 4 | 196 | - | 2% | 0.04% |
| 3 | Tube #2 | 4 | - | 156 | 0.04% | 0.001% |

## 11. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use.  It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or “edge effects” have not been observed with this assay.

## 12. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4ºC storage.

13.3 Add 50 µL of all sample or standard to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

*Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:*

| Mode: | Kinetic |
|---|---|
| Wavelength: | 600 nm |
| Time: | up to 15 min |
| Interval: | 20 sec - 1 min |
| Shaking: | Shake between readings |

*Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.*

13.9 Analyze the data as described below.

## 13. CALCULATIONS

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 **Create a standard curve** by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

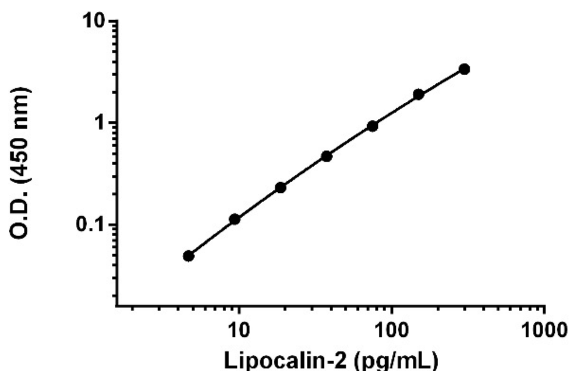
*Note*: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted **absorbance values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

## 14. TYPICAL DATA

**TYPICAL STANDARD CURVE** – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.




| Standard Curve Measurements | | | |
|---|---|---|---|
| Conc. (pg/mL) | O.D. 450 nm | | Mean O.D. |
| | 1 | 2 | |
| 0 | 0.062 | 0.060 | 0.061 |
| 4.688 | 0.112 | 0.111 | 0.111 |
| 9.375 | 0.176 | 0.174 | 0.175 |
| 18.75 | 0.297 | 0.289 | 0.293 |
| 37.50 | 0.531 | 0.533 | 0.532 |
| 75.00 | 0.992 | 0.987 | 0.990 |
| 150.0 | 1.971 | 1.964 | 1.967 |
| 300.0 | 3.433 | 3.436 | 3.434 |

**Figure 1.** Example of Lipocalin-2 standard curve. The Lipocalin-2 standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

## 15. TYPICAL SAMPLE VALUES

**SENSITIVITY –**

The calculated minimal detectable dose (MDD) is 1.1 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=10) and adding 2 standard deviations then extrapolating the corresponding concentrations.

**RECOVERY –**

Three concentrations of Lipocalin-2 were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

| Sample Type | Average % Recovery | Range (%) |
|---|---|---|
| 0.02% Mouse Serum | 109 | 106-110 |
| 0.02% Mouse Plasma - Citrate | 109 | 98-116 |
| 0.02% Mouse Plasma (platelet poor EDTA) | 107 | 87-120 |
| 0.2% Mouse Urine | 105 | 100-107 |
| Cell Culture Media | 104 | 102-107 |
| Cell Culture Media (Serum Free) | 99 | 98-103 |

## LINEARITY OF DILUTION –

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Lipocalin-2 was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

| Dilution Factor | Interpolated value | 0.05% Mouse Serum | 0.05% Mouse Plasma (Citrate) | 0.05% Mouse Plasma (EDTA) Platelet Poor | 0.2% Mouse Urine | 5% J774A.1 Stimulated Supernatant |
|---|---|---|---|---|---|---|
| Undiluted | pg/mL | 130.2 | 295.0 | 280.7 | 226.7 | 258.9 |
| | **% Expected value** | **100** | **100** | **100** | **100** | **100** |
| 2 | pg/mL | 65.3 | 145.4 | 132.6 | 112.4 | 133.0 |
| | **% Expected value** | **100** | **99** | **94** | **99** | **103** |
| 4 | pg/mL | 33.3 | 71.7 | 65.8 | 57.1 | 63.0 |
| | **% Expected value** | **102** | **97** | **94** | **101** | **97** |
| 8 | pg/mL | 17.0 | 36.9 | 33.7 | 27.9 | 30.8 |
| | **% Expected value** | **104** | **100** | **96** | **98** | **95** |
| 16 | pg/mL | 8.20 | 18.4 | 17.0 | 14.36 | 15.2 |
| | **% Expected value** | **101** | **100** | **97** | **101** | **94** |

NL – Non-Linear

## PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of mouse serum within the working range of the assay.

| | Intra-Assay | Inter-Assay |
|---|---|---|
| n= | 5 | 3 |
| CV (%) | 3.7 | 6.1 |

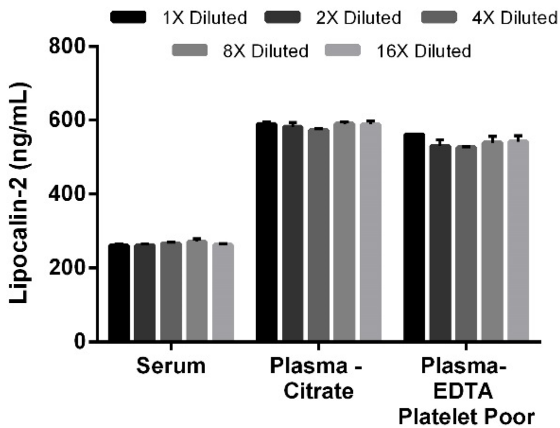



**Figure 2.** Interpolated concentrations of Lipocalin-2 in mouse serum, plasma (citrate), and platelet poor plasma (EDTA). The concentrations of Lipocalin-2 were measured in duplicate and interpolated from the Lipocalin-2 standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Lipocalin-2 concentration was determined to be 265.2 ng/mL in mouse serum, 583.8 ng/mL in mouse plasma (Citrate), and 535.2 ng/mL in platelet poor mouse plasma (EDTA).

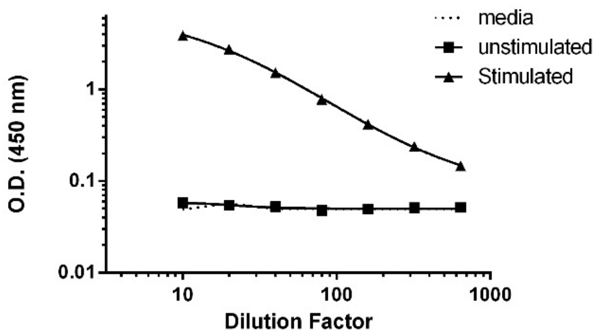



**Figure 3.** Comparison of secreted Lipocalin-2 in media, unstimulated and PMA/PHA-stimulated J774A.1 cells. J774A.1 cells were grown in the absence (unstimulated) or presence of Phorbol Myristate Acetate (PMA) and phytohemagglutinin (PHA) (stimulated) for 3 days. Raw data from duplicate measurements are plotted (mean +/- SD, n=2).

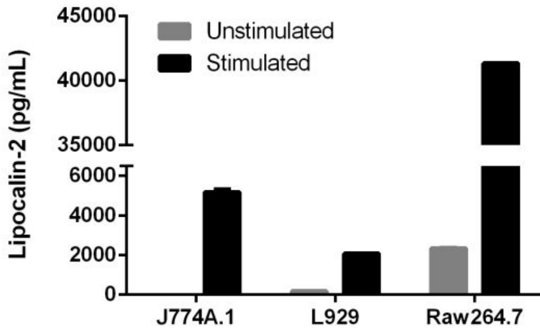



**Figure 4.** Comparison of secreted Lipocalin-2 in mouse cell lines with or without stimulation. L929 and J774A.1 cells were grown in the absence (unstimulated) or presence of Phorbol Myristate Acetate (PMA) and phytohemagglutinin (PHA) (stimulated) for 3 days. RAW264.7 were grown in the absence (unstimulated) or presence of LPS (stimulated) for 2 days. Cell Culture supernatant was diluted to within the range of the assay, 5-fold (L929 and J774A.1) or 2,000-fold (RAW264.7). Measured values were interpolated from the Lipocalin-2 Standard Curve diluted in Sample Diluent NS and corrected for dilution factor. Mean of duplicate values +/-SD are graphed. Lipocalin-2 was undetectable in media.

## 16. ASSAY SPECIFICITY

This kit recognizes both native and recombinant mouse Lipocalin-2 protein in serum, plasma, urine and cell culture supernatant.

Cell and tissue extract samples have not been tested with this kit.

## 17. SPECIES REACTIVITY

This kit recognizes mouse Lipocalin-2 protein.

Other species reactivity was determined by measuring 1,000-fold diluted serum samples of various species, interpolating the protein concentrations from the mouse standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in mouse serum assayed at the same dilution.

Reactivity < 3% was determined for the following species:

- Human
- Rat
- Hamster
- Guinea Pig
- Rabbit
- Dog
- Goat
- Pig
- Cow

Please contact our Technical Support team for more information

## 18. TROUBLESHOOTING

| Problem | Cause | Solution |
|---|---|---|
| Difficulty pipetting lysate; viscous lysate. | Genomic DNA solubilized | Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction. |
| Poor standard curve | Inaccurate Pipetting | Check pipettes |
| | Improper standard dilution | Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing |
| Low Signal | Incubation times too brief | Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation |
| | Inadequate reagent volumes or improper dilution | Check pipettes and ensure correct preparation |
| | Incubation times with TMB too brief | Ensure sufficient incubation time until blue color develops prior addition of Stop solution |
| Large CV | Plate is insufficiently washed | Review manual for proper wash technique. If using a plate washer, check all ports for obstructions. |
| | Contaminated wash buffer | Prepare fresh wash buffer |
| Low sensitivity | Improper storage of the ELISA kit | Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB Development solution protected from light. |
| Precipitate in Diluent | Precipitation and/or coagulation of components within the Diluent. | Precipitate can be removed by gently warming the Diluent to 37ºC. |

## 19. NOTES

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**UK, EU and ROW**
Email: technical@abcam.com | Tel: +44-(0)1223-696000

**Austria**
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

**France**
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

**Germany**
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

**Spain**
Email: soportecientifico@abcam.com | Tel: 911-146-554

**Switzerland**
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

**US and Latin America**
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

**Canada**
Email: ca.technical@abcam.com | Tel: 877-749-8807

**China and Asia Pacific**
Email: hk.technical@abcam.com | Tel: 400 921 0189 / +86 21 2070 0500

**Japan**
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp

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