# KRIBIOLISA® Alemtuzumab (LEMTRADA™) ELISA

REF : KBI1012

Ver 5.4

RUO

Enzyme Immunoassay for the quantitative determination of Alemtuzumab in human serum and plasma

| | | | |
|---|---|---|---|
| RUO | For Research Use Only | REF | Catalog Number |
| | Store At | LOT | Batch Code |
| | Manufactured By | | Biologi isk |
| | Expiry D | | Consu rating Instructions |

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REF KBI1012 Σ 96 tests

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**Introduction:**

Alemtuzumab is a drug used in the treatment of chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL) and T-cell lymphoma under the trade names Campath, MabCampath and Campath-1H, and in the treatment of multiple sclerosis as Lemtrada. It is also used in some conditioning regimens for bone marrow transplantation, kidney transplantation and islet cell transplantation. It is a monoclonal antibody that binds to CD52, a protein present on the surface of mature lymphocytes, but not on the stem cells from which these lymphocytes are derived. After treatment with Alemtuzumab, these CD52-bearing lymphocytes are targeted for destruction. MabCampath was withdrawn from the markets in the US and Europe in 2012 to prepare for a higher-priced relaunch of Lemtrada aimed at multiple sclerosis.

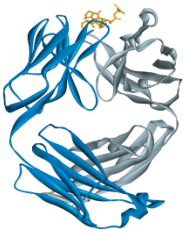


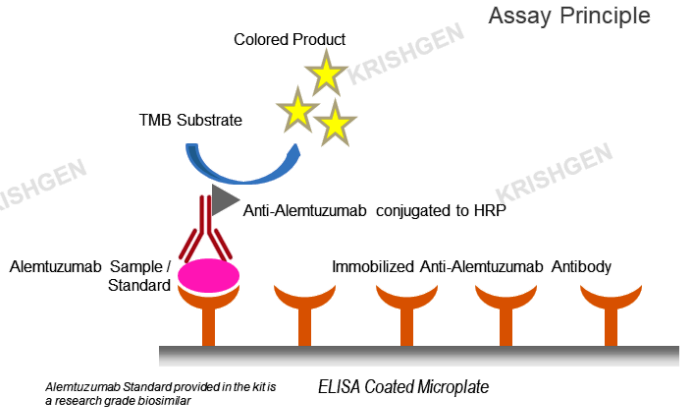
Image Courtesy: By Fvasconcellos 02:03, 28 May 2007

**Intended Use:**

The KRIBIOLISA® Alemtuzumab (LEMTRADA™) ELISA is used as an analytical tool for quantitative determination of Alemtuzumab in human serum and plasma.

**Principle:**

The method employs the quantitative sandwich enzyme immunoassay technique. Antibodies to Alemtuzumab are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Alemtuzumab present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated anti-Alemtuzumab antibody is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution 3, 3', 5, 5' Tetra Methyl Benzidine (TMB) is added to microwells and color develops proportionally to the amount of Alemtuzumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.




**PRINCIPLE OF THE KRIBIOLISA ALEMTUZUMAB (LEMTRADA™) ELISA**

**Materials Provided:**

| Part | Description | Qty |
|---|---|---|
| Anti-Alemtuzumab Coated Microtiter Plate | 96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Alemtuzumab monoclonal antibody | 1 x 96 wells |
| Alemtuzumab Standard | Recombinant Alemtuzumab in a buffered protein base with preservative thiomersal <0.01% (lyophilized, concentration - 1 ug/ml) | 2 vials |
| Anti-Alemtuzumab:HRP Conjugate | Anti-Alemtuzumab conjugated to Horseradish Peroxidase with protein stabilizer and preservatives 0.02% methylisothiazolinone and 0.02% bromonitrodioxane | 12 ml |
| (1X) Sample Diluent | Buffered protein base with preservative thiomersal <0.01% | 50 ml |
| (1X) Standard Diluent | Buffered protein base with 1:100 dilution human serum and preservative thiomersal <0.01% | 10 ml |
| (20X) Wash Buffer | 20-fold concentrated solution of buffered surfactant with preservative thiomersal <0.01%. May turn yellow over time | 25 ml |
| TMB Substrate | Stabilized chromogen | 12 ml |
| Stop Solution | 0.73M Phosphoric Acid | 12 ml |

| | | |
|---|---|---|
| Instruction Manual | | 1 no |

**Materials to be provided by the End-User:**

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Timer.
7. Absorbent Paper.

**Handling/Storage:**

1. All reagents should be stored at 2ºC to 8ºC for stability.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

**Health Hazard Warnings:**

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.



**Sample Preparation and Storage:**

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at -20°C.

For Cell Culture Supernatant **–** If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80$^0$C. Avoid repeated freeze-thaw cycles.

For Serum - Samples have to be diluted 1:100 (v/v), e.g. 1 ul sample + 99 ul sample diluent prior to assay. The samples may be kept at 2-8°C for up to three days. Long-term storage requires -20°C.

For Plasma - Samples have to be diluted 1:100 (v/v), e.g. 1 ul sample + 99 ul sample diluent prior to assay. The samples may be kept at 2-8°C for up to three days. Long-term storage requires -20°C.

**Preparation Before Use:**
Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

**Reagent Preparation (all reagents should be diluted immediately prior to use):**

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. To make Wash Buffer (1X); dilute 25 ml of 20X Wash Buffer in 475 ml of DI water.
4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 1 ml of Standard Diluent (1X) to obtain a concentration of 1 ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 320 ul of original **Standard (1 ug/ml)** with 180 ul of Standard Diluent (1X) to generate a **640 ng/ml Standard Solution**. Prepare further **Standards** by serially diluting the Standard Solution as per the below table. Use the Standard Diluent (1X) as the Zero Standard (Standard No.0).

| Standard Concentration | Standard Vial | Dilution Particulars |
|---|---|---|
| 1 ug/ml | Reconstituted Standard | Lyophilized Standard provided in the Kit + + Reconstitute in 1 ml of Standard Diluent (1X) |
| 640 ng/ml | Standard No.7 | 320 ul Reconstituted Standard (1 ug/ml) + 180 ul Standard Diluent (1X) |
| 320 ng/ml | Standard No.6 | 250 ul Standard No.7 + 250 ul Standard Diluent (1X) |
| 160 ng/ml | Standard No.5 | 250 ul Standard No.6 + 250 ul Standard Diluent (1X) |
| 80 ng/ml | Standard No.4 | 250 ul Standard No.5 + 250 ul Standard Diluent (1X) |
| 40 ng/ml | Standard No.3 | 250 ul Standard No.4 + 250 ul Standard Diluent (1X) |
| 20 ng/ml | Standard No.2 | 250 ul Standard No.3 + 250 ul Standard Diluent (1X) |
| 10 ng/ml | Standard No.1 | 250 ul Standard No.2 + 250 ul Standard Diluent (1X) |
| 0 ng/ml | Standard No.0 | Only Standard Diluent (1X) |

Use the Standards immediately upon reconstitution. Discard balance standard after use. Do not store them for further experiments

**Procedural Notes:**

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Alemtuzumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Alemtuzumab present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Alemtuzumab concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
3. Avoid assay of Samples containing sodium azide ($NaN_3$), as it could destroy the HRP activity resulting in under-estimation of the amount of Alemtuzumab.
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

**Assay Procedure:**

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay. All steps must be performed at 37°C.

2. Add **100 ul** of **Standards** or **diluted Samples** into the respective wells.

3. Cover the plate and incubate for 60 minutes at 37°C.

4. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

5. Add **100 ul** of **Anti-Alemtuzumab:HRP Conjugate** into each well.

6. Cover the plate and incubate for 60 minutes at 37°C.

7. Aspirate and wash plate 4 times with **Wash Buffer (1X)** same as in step 4.

8. Add **100 ul** of **TMB Substrate** in each well.

9. Incubate the plate at 37°C for 30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

10. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.

11. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

**Calculation of Results:**

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Alemtuzumab concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the

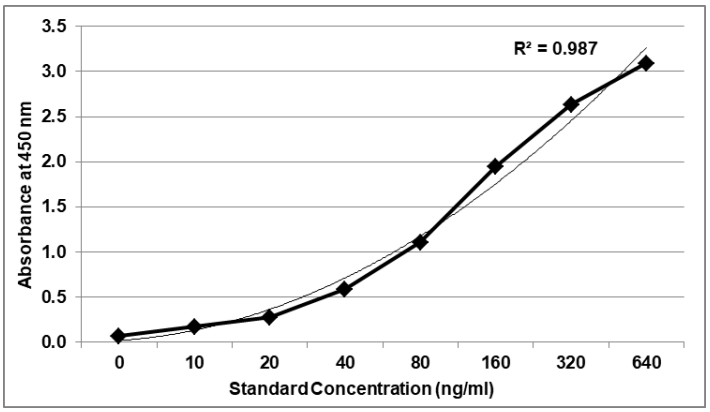
Alemtuzumab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit is best recommended for automated results.

**Note:**
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 640 ng/ml standard.

**Typical Data (representative only)**

| Standard provided (ng/ml) | Abs A | Abs B | Mean Abs | Interpolated Concentration | % Interpolated Concentration against Actual Concentration |
|---|---|---|---|---|---|
| 0 | 0.069 | 0.067 | 0.068 | -- | -- |
| 10 | 0.169 | 0.177 | 0.173 | 11.3 | 112.8 |
| 20 | 0.302 | 0.255 | 0.278 | 19.6 | 98.2 |
| 40 | 0.620 | 0.549 | 0.585 | 40.9 | 102.2 |
| 80 | 1.172 | 1.046 | 1.109 | 78.1 | 97.6 |
| 160 | 1.972 | 1.920 | 1.946 | 163.1 | 101.9 |
| 320 | 2.619 | 2.648 | 2.633 | 315.5 | 98.6 |
| 640 | 3.091 | 3.090 | 3.090 | 644.5 | 100.7 |

**Typical Graph (representative only)**



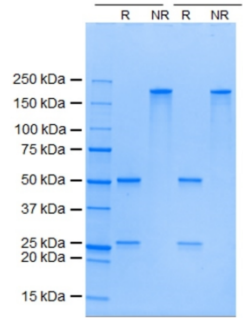

**Quality Control:**

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

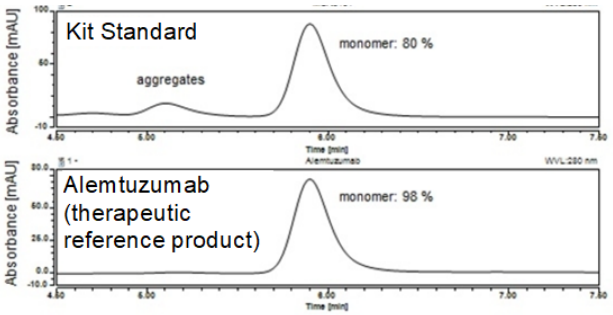
**Performance Characteristics of the Kit:**

This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

**Specificity:**
The capture antibody used in the kit is specific for Alemtuzumab (CAMPATH/LEMTRADA) and is a monovalent anti-idiotypic human recombinant Fab (lambda light chain) expressed in E. coli that specifically recognizes humanized Alemtuzumab The intrinsic affinity of the monovalent form of this antibody is KD=0.2 nM as measured by real time, label-free molecular interaction analysis on immobilized Alemtuzumab. The kit standard used is a recombinant human IgG1 kappa antibody with variable regions from the therapeutic antibody Campath-1H (Lemtrada). The standadard has been validated by HPLC against the referenced therapeutic product.




**Purity Assesment by SDS Page**




**Sec of Standard with Referenced Product**

**Sensitivity:**
**Limit Of Detection:** It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD.
10 replicates of '0' standards were evaluated and the LOD was found to be less than 10 ng/ml.

**Linearity:**
Standards provided in the kit will be used for measuring the dilutional linearity range of Alemtuzumab present in matrix. It was observed that the sample matrix - serum/plasma gave optimal recoveries at 1:100 dilution.

| Human Serum Dilution | Standards provided (ng/ml) | Abs | Interepolated Concentration | % Interpolated Concentration against Actual Concentration |
|---|---|---|---|---|
| Serum 1:10 dilution | 0 | 0.069 | -- | -- |
| | 640 | 3.271 | Abs* beyond Std Range | * |
| Serum 1:100 dilution | 0 | 0.059 | -- | -- |
| | 640 | 3.016 | 551.7 | 86.2 |
| Serum 1:1000 dilution | 0 | 0.060 | -- | -- |
| | 640 | 2.944 | 484.5 | 75.7 |
| Serum 1:2000 dilution | 0 | 0.058 | -- | -- |
| | 640 | 2.834 | 408.4 | 63.8 |
| Serum 1:5000 dilution | 0 | 0.057 | -- | -- |
| | 640 | 3.107 | 670.6 | 104.8 |

***Abs* absorbance was beyond the absorbance value of the graph, hence interpolation of the concentration not possible***

| Human Plasma Dilution | Standards provided (ng/ml) | Abs | Interepolated Concentration | % Interpolated Concentration against Actual Concentration |
|---|---|---|---|---|
| Plasma 1:10 dilution | 0 | 0.051 | -- | -- |
| | 640 | 3.184 | 825.2 | 128.9 |
| Plasma 1:100 dilution | 0 | 0.059 | -- | -- |
| | 640 | 2.923 | 467.9 | 73.1 |
| Plasma 1:500 dilution | 0 | 0.060 | -- | -- |
| | 640 | 2.920 | 465.6 | 72.8 |
| Plasma 1:1000 dilution | 0 | 0.066 | -- | -- |
| | 640 | 2.998 | 533.2 | 83.3 |
| Plasma 1:2000 dilution | 0 | 0.058 | -- -- | -- |
| | 640 | 2.994 | 529.3 | 82.7 |
| Plasma 1:5000 dilution | 0 | 0.062 | | -- |
| | 640 | 2.899 | 450.3 | 70.4 |

**Precision:**
Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (10ng/ml), medium (80ng/ml) and high (640ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

| Pool | Intra Assay %CV | Inter Assay %CV |
|---|---|---|
| Low | <10% | <10% |
| Medium | <5% | <5% |
| High | <5% | <5% |

**Note:**
For researchers and clinical companies looking to use the kit for demonstration and estimation of their own developed biosimilar Alemtuzumab with the referenced innovator product, it is recommended to first run similarity assay using the kit provided Standard. Subsequently, you may use your internal biosimilar as standard in the assay once the parameters are established.

In case the demonstrated variance is beyond the defined quality limits when using your own biosimilar, we would recommend to use the kit provided standard only. In case the recoveries obtained are not as per the desired results, please connect with us (email: sales1@krishgen.com) to help you optimize the assay using our own differently formulated diluents to best optimize on the kit.

**Safety Precautions:**

- **This kit is For Research Use only.** Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (<0.1% w/v) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials maybe derived from **human body fluids** or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
  - Do not smoke, eat or drink while handling kit material
  - Always use protective gloves
  - Never pipette material by mouth
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.

- In any case GLP should be applied with all general and individual regulations to the use of this kit.

**References:**

Alemtuzumab compared with chlorambucil as first-line therapy for chronic lymphocytic leukemia P Hillmen, AB Skotnicki, T Robak, B Jaksic… - Journal of Clinical …, 2007 - ascopubs.org

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## SCHEMATIC ASSAY PROCEDURE

1. Remove all components, 30 minutes before adding into the assay plate.

Components (2-8ºC) → 30 mins RT Before Use → 

Thaw at Room Temperature (18-24ºC) → USE NOW

2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

Kit Components → AVOID → COOL (2-8ºC) → THAW (22±4ºC) → THAW (22±4ºC) → COOL (2-8ºC) → AVOID → NO CHANGE IN RESULTS

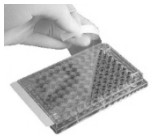
3.  Pipette **100 ul Standards** / **diluted Samples** into the respective wells.

4. Cover plate  and incubate for 

at 37°C.

5.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**

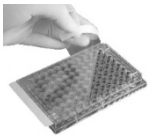
6.  Pipette **100 ul Anti-Alemtuzumab:HRP** into each well.

7. Cover plate  and incubate for 

at 37°C.

8.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**

9.  Pipette **100 ul TMB Substrate** into each well.

10. Cover plate  and incubate for 

at 37°C.

11.  Pipette **100 ul Stop Solution** into each well.

12. Read absorbance at 450 nm with a  microplate reader within 

of stopping reaction.

**Typical Example of a Work List**

| Well # | Contents | Absorbance at 450nm | Mean Absorbance | ng/ml Alemtuzumab equivalent |
|---|---|---|---|---|
| 1A<br>2A | zero std<br>zero std | | | |
| 1B<br>2B | 10 ng/ml<br>10 ng/ml | | | |
| 1C<br>2C | 20 ng/ml<br>20 ng/ml | | | |
| 1D<br>2D | 40 ng/ml<br>40 ng/ml | | | |
| 1E<br>2E | 80 ng/ml<br>80 ng/ml | | | |
| 1F<br>2F | 160 ng/ml<br>160 ng/ml | | | |
| 1G<br>2G | 320 ng/ml<br>320 ng/ml | | | |
| 1H<br>2H | 640 ng/ml<br>640 ng/ml | | | |
| 3A<br>4A | Sample | | | |
| 3B<br>4B | Sample | | | |

**LIMITED WARRANTY**

Krishgen Biosystems Private Limited does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the Products; against defects in products or components not manufactured by Krishgen Biosystems Private Limited, or against damages resulting from such non-Krishgen Biosystems Private Limited made products or components. Krishgen Biosystems Private Limited passes on to customer the warranty it received (if any) from the maker thereof of such non Krishgen made products or components. This warranty also does not apply to Products to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Krishgen Biosystems Private Limited.

THIS WARRANTY IS EXCLUSIVE. The sole and exclusive obligation of Krishgen Biosystems Private Limited shall be to repair or replace the defective Products in the manner and for the period provided above. Krishgen Biosystems Private Limited shall not have any other obligation with respect to the Products or any part thereof, whether based on contract, tort, and strict liability or otherwise. Under no circumstances, whether based on this Limited Warranty or otherwise, shall Krishgen Biosystems Private Limited be liable for incidental, special, or consequential damages.

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**SYMBOLS KEY**

| | |
|---|---|
| MTP | Anti-Alemtuzumab Coated Microtiter Plate (12 wells) |
| STD | Alemtuzumab Standard, Lyophilized |
| HRP CONJ | Alemtuzumab:HRP Conjugate |
| 1X SAMP DIL | Sample Diluent |
| 1X STD DIL | Standard Diluent |
| 20X WASH BUF | Wash Buffer |
| SUB TMB | Substrate |
| SOLN STOP | Solution |
| | Consult Instructions for Use |
| REF | Catalog Number |
| | Expiration Date |
| | Storage Temperature |