INTENDED USE

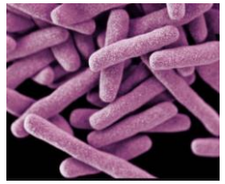
The **Human Anti-Tuberculosis (Bacillus Calmette-Guerin/ BCG) IgG** ELISA Kit is an immunoassay suitable for quantifying IgG antibody activity specific for BCG in serum or plasma of vaccinated, immunized and/or infected hosts.

This immunoassay is suitable for:

- Determining **immune status** relative to non-immune controls;
- Assessing efficacy of **vaccines**, including dosage, adjuvantcy, route of immunization and timing;
- Qualifying and standardizing vaccine batches & protocols

The assay is for research use only (RUO) and is not intended nor validated for diagnosing Tuberculosis disease.

GENERAL INFORMATION



Mycobacterioses (tuberculosis, leprosy, atypical mycobacterioses, paratuberculosis, and perhaps Crohn's Disease) are diseases of humans and animals with the largest diffusion on earth. The infectious agents of tuberculosis are acid-resistant rod-like bacteria of the family Mycobacteriaceae, genus Mycobacterium. The main cause of TB is Mycobacterium tuberculosis, a small, aerobic, nonmotile bacillus. The high lipid content of this pathogen accounts for many of its unique clinical characteristics. If a Gram stain is performed, MTB either stains very weakly "Gram-positive" or does not retain dye as a result of the high lipid and mycolic acid content of its cell wall. The only currently available vaccine as of 2012 is bacillus Calmette–Guérin (BCG with live attenuated bacteria) which, while it is effective against disseminated disease in childhood, confers inconsistent protection against contracting pulmonary TB. Nevertheless, it is the most widely used vaccine worldwide, with more than 90% of all children being vaccinated.

A number of new TB vaccines are currently in phase I and II clinical trials. MVA85A (modified vaccinia Ankara 85A, Oxford University) is a subunit vaccine to BCG, which uses the attenuated MVA as a vaccine delivery platform to present antigen 85A to the immune system.

BCG vaccines: Pacis® BCG, made from the Montréal (Institut Armand-Frappier) strain (Dianon/Urocor). BCG vaccine Danish strain 1331 (Statens Serum Institut, Denmark), Tokyo BCG TY-1002, Tokyo 172 substrain of Pasteur BCG (Japan, BCG Labs), Moscow BCG 254-2; BCG vaccine Glaxo 1077 strain (Sanofi). All vaccine use attenuated M. Bovis strains.

PRINCIPLE OF THE TEST

The Anti-BCG IgG/IgM ELISA kits are based on the binding of antibodies (IgG/IgM) in samples to the purified BCG antigens immobilized on the microwells. Bound antibody is detected by anti-human IgG or IgM-HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color is developed by the HRP substrate, which is directly proportional to the amount of anti-BCG IgG or IgM present in the sample. Stop Solution is added to terminate the reaction, and Absorbance is then measured using an ELISA reader at 450nm. The presence of antibody (IgG/IgM) in samples is determined relative to anti-BCG IgG/IgM Calibrators and Controls.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8^{O}C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at 4^{o}C for long term and ambient temp. for short term.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample/Conjugate Diluent** and store at 2-8^{o}C until the kit lot expires or is used up.
Anti-Human IgG-HRP Conjugate Concentrate (100x) Part: H-HuG.2a11, 0.15ml	Peroxidase conjugated anti-human IgG in buffer with detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample/Conjugate Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8^{o} C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
BCG Coated Strip Plate	990311	8-well strips (12)	Coated with purified recombinant BCG, and post-coated with stabilizers.
Anti-BCG IgG Calibrators			
3 U/ml 10 U/ml 30 U/ml	990312B 990312C 990312D	1.0 ml 1.0 ml 1.0 ml	Three (3) vials, each containing anti-BCG IgG; in buffer with antimicrobial as stabilizers.
Anti-BCG IgG Positive Control	990313 PC .	1.0 ml	Anti-BCG; in buffer with antimicrobial as stabilizers. [Value range is on the label]
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Human IgG HRP Concentrate.
- Stock bottle to store diluted Wash Solution; 0.2 to 1L.
- ELISA plate reader at 450 nm wavelength and ELISA plate washer

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Antibody Stability

An initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for years, stored refrigerated or frozen. Further dilution into WSD, which provides the lowest assay background, can be made from the initial dilution, as required.

Assay Design

Review Interpretation of Results (p5-7) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for non-immune samples should be lower than the **3 U/ml Calibrator**. This is usually 1:100 or greater dilution for human serum with normal levels of IgG and IgM.
- Run the **Anti-BCG IgG Positive Control**; value range is on the vial label.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required. Blank OD should be <0.3.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **30 U/ml** should give a high signal (>1.5 OD); **3 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).

Plate Set-up

Bring all reagents to room temperature (18-30^{o} C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Human IgG HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

3. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

4. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

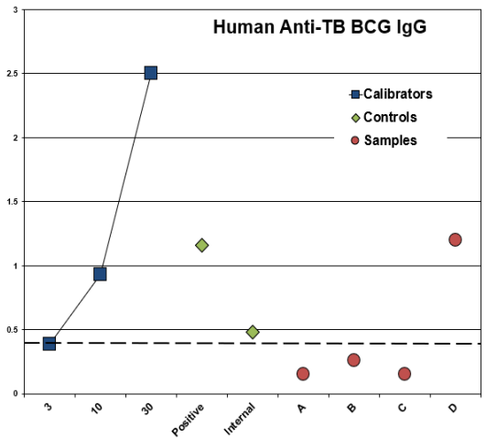
INTERPRETATION OF RESULTS

Method A. Antibody Activity Threshold Index

Compare Samples to **3 U/ml Calibrator** or **Internal Control**

= Positive/Negative Cut-off.

Example:



Results

The **sensitivity** of the assay to detect anti-BCG IgG, from either natural infection or vaccination, is controlled so that the **3 U/ml Calibrator** represents a threshold OD for most true positives in human serum diluted to 1:100 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of an anti-BCG antibody, derived from BCG immunization, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.
3 U/ml: a 'Cut-off' line has been drawn to indicate a threshold distinguishing between **Positive/Negative**. **Note**: This is not a clear-cut threshold, rather a low OD area that could represent either low positives or high background negatives.

Positive Control – anti-BCG serum; value range is on the label. This control can be used to assess reproducibility and normalize between-assay values.

Internal Control – a true positive from an immune host that represents the investigator's experience in distinguishing low positive from negative samples (not in kit). This should be run in each assay to supplement the 3 U/ml Calibrator for Positive/Negative discrimination purposes.

Samples A,B,C,D – 3 samples (A, B, C) are negative: below the threshold; 1 sample (D) is positive: clearly above the threshold.

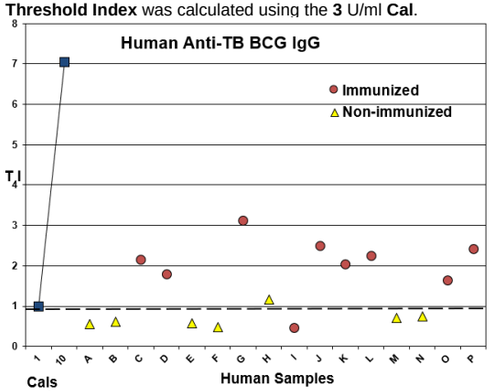
The **3 U/ml** Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative (see p6):

- Divide each Sample net OD by the 3 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

ASSAY PERFORMANCE

Example:

Human Serum IgG
A panel of sera from humans immunized with the BCG vaccine (childhood) or not was tested for anti-BCG IgG (1:100 dilution). **Threshold Index** was calculated using the **3** U/ml **Cal**.



Results

Non-immunized: six samples were negative (below 1.0 threshold); one serum (H) was borderline.

Immunized: eight samples were positive; one serum (I) was negative.

Notes:

1. **Positives** may also be due to prior encounter with the bacteria or non-BCG proteins with common epitopes, or may be an aspect of the innate immune repertoire.
2. The **sensitivity** of the assay may be adjusted by changing the sample dilutions:
   a) **increase dilution** (e.g., 1:200) to lower the signals of borderline positives to negative
   b) **decrease dilution** (e.g., 1:50) to convert borderline samples to positive. With the latter, negative values may increase, so an alternative threshold should be considered using known negatives to develop a **Positive Index (**see below) or use an **Internal Control** (Page 5).

B. Positive Index
Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.
2. Divide each sample net OD by the Positive Index. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

A sample value would be **Positive** if significantly above the value of the pre-immune serum sample or a suitably determined non-immune panel or pool of samples, tested at the same sample dilution.

This calculation also **quantifies** the positive Antibody Activity level, assigning a higher value to samples with higher Antibody Activity, and vice versa.

INTERPRETATION OF RESULTS (cont)

Method C. Titers from Sample Dilution Curves

The titer of elevated antibody activity calculated from a dilution curve of each sample is recommended as the most accurate quantitative method. Best precision can be obtained using the following guidelines:

1. Use an OD value Index in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce signals higher and lower than the selected index. With accurate diluting, duplicates may not be required if at least 4 dilutions are run per sample.
3. A 5-fold dilution scheme is useful to efficiently cover a wide range which produces ODs both above and below 1.0 OD. The dilution scheme can be tightened to 3-fold or 2-fold for more precise comparative data.
4. The Positive and Sensitivity Control values can be used to normalize inter-assay values.

Calculations

1. On a log scale of inverse of Sample Dilution as the x-axis, plot the OD values of the two dilutions of each positive sample having ODs above and below the OD value of the Index (arbitrary or selected Calibrator).
2. From a point-to-point line drawn between the two sample ODs, read the dilution value (x-axis) corresponding to the OD of the selected Index

= IgG Antibody Activity Units

PRODUCT SPECIFICATIONS

Specificity

The coated BCG antigens are non-specified, complex mixture of many tuberculosis antigens; the kit will detect antibodies to various strains of mycobacterium. The Anti-human IgG HRP conjugate detects IgG, and does not react with IgM, IgA or IgE.

Sensitivity
The BCG-coated plate and Anti-Human IgG HRP concentration are optimized to differentiate anti-BCG IgG from background (non-antibody) signal with human serum samples diluted 1:100.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested

Instruction Manual No. M-990-330-THG

Recombivirus™ Human Anti-Tuberculosis BCG (Bacillus Calmette-Guerin) IgG ELISA Kit

Catalog No. 990-330-THG

For the Detection of Anti-BCG IgG in Human Serum or Plasma

For research use only, not for diagnostic or therapeutic use.

ALPHA DIAGNOSTIC INTERNATIONAL

India Contact:

Life Technologies™
Your Molecular & Cell Technology Partner

Life Technologies (India) Pvt. Ltd.
306, Aggarwal City Mall, Opposite M2K Pitampura,
Delhi – 110034 (INDIA).
Ph: +91-11-42208000, 42208111, 42208222
Mobile: +91-9810521400
Fax: +91-11-42208444
Email: customerservice@lifetechindia.com
Web: www.lifetechindia.com

ELISA Kit Components	Amount	Part
BCG Coated Strip Plate	8-well strips (12)	990311
Anti- BCG Positive Control	1.0 ml	990313PC
Anti- BCG Calibrator 3 U/ml	1.0 ml	990312B
Anti- BCG Calibrator 10 U/ml	1.0 ml	990312C
Anti- BCG Calibrator 30 U/ml	1.0 ml	990312D
Anti-Human IgG HRP Conjugate (100X)	0.15 ml	H-HuG.2a11
Sample Diluent (20x)	10 ml	SD20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	990-330-THG