

QuantiFERON[®]-CMV FAQs

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Test Principle

The QuantiFERON[®] -CMV assay is an *in vitro* research test that detects CD8+ T cell immunity to human cytomegalovirus infection (HCMV). It uses human whole blood, with patented assay technology based on the measurement of Interferon-gamma (IFN- γ) secreted from stimulated T-cells previously exposed to HCMV. The QuantiFERON[®] -CMV assay is a straightforward laboratory test that involves 4 simple steps:

1. Collection of blood into QuantiFERON[®] -CMV Blood Collection Tubes.
2. Overnight incubation at 37°C. HCMV infected patients' blood cells will produce IFN- γ .
3. Detection of released IFN- γ in harvested plasma using a quick and easy ELISA.
4. Analysis of data using the QuantiFERON[®] -CMV Analysis Software.

Some of the Frequently Asked Questions relating to the assay are listed below. The answers provided act as a guide only. We recommend that the QuantiFERON[®] -CMV Package Insert be used as the reference for the test procedure, as well as for other enquiries relating to the use or performance of the assay.

Step 1: Blood Collection

The blood hasn't reached the black mark on the side of the Blood Collection Tube. Is this important?

The mark on the side of the tubes indicates the 1 mL fill volume. QuantiFERON[®] -CMV Blood Collection Tubes have been validated for volumes ranging from 0.8 to 1.2 mL. If the level of blood in any tube is not close to the indicator mark, it is recommended to obtain another blood sample.

How important is the tube mixing process?

The antigen mixing process ensures even distribution of stimulating antigens to allow white blood cells to ingest and process antigen for presentation to T-cells, thus leading to IFN- γ secretion. It is a very important step in the QuantiFERON[®] -CMV assay and poor mixing will lead to low and incorrect results.

Mix the tubes by vigorously shaking the tubes 10 times ensuring that the entire surface of the tube has been coated with blood. Thorough mixing is required to ensure complete mixing of the blood with the tube's contents. Causing the blood to froth will not adversely affect the performance of the test. Universal blood handling precautions should be used.

A demonstration video can be viewed on our website at: www.QuantiFERON.com

Can the blood collection tubes be transported lying down?

Yes. QuantiFERON[®] -HCMV Collection Tubes can be transported lying down only after the tube mixing step has been done. The tubes should be mixed again immediately prior to being placed upright in the 37°C incubator.

At what temperature can the blood be transported to another site, or held prior to incubation at 37°C?

Blood should be held and transported at Room Temperature (17°C to 27°C). Do not refrigerate the blood or place on ice.

Step 2. Blood Incubation and Plasma Harvesting

What if 37°C incubation starts more than 16 hours after the time of blood collection?

The Package Insert specifies that the 37°C blood incubation must commence within 16 hours of collection. Blood samples incubated more than 16 hours after collection are likely to exhibit a decreased IFN- γ response due to cellular breakdown (death), leading to loss of sensitivity and inaccurate results.

Can I incubate the blood collection tubes lying down?

QuantiFERON[®] -HCMV Blood Collection Tubes must be kept upright during incubation at 37°C.

Do I have to centrifuge the tubes before I can harvest the plasma?

While it is recommended to centrifuge the tubes to assist with harvesting, it is possible to harvest the plasma from the tubes without centrifugation. Additional care is required to remove the plasma without disturbing the cells.

Do I have to centrifuge the tubes immediately after removal from incubator?

QuantiFERON[®] -HCMV Blood Collection Tubes may be held between 2°C and 27°C for up to 3 days before centrifugation or harvesting.

The gel plug hasn't moved during centrifugation. What should I do?

After incubation of tubes at 37°C, the plasma is separated from the cells by centrifuging for 15 minutes at 2000 - 3000 RCF (g). The gel plug should move to separate the cells from the plasma. If this does not occur, the tubes should be re-centrifuged at a higher speed.

The plasma doesn't appear the colour it normally does. Is this OK?

Plasma from the QuantiFERON[®] -CMV Blood Collection Tubes can appear more red than usual but this is normal. It should be noted that the colour of plasma, even those without any red blood cell contamination, can vary from almost colourless to shades of yellow/pale brown; some plasma samples even have an opaque character. These qualities have not been found to affect QuantiFERON[®] -CMV results.

Do I require a Class II Biohazard Cabinet in which to perform plasma harvesting?

Ideally, all work with blood should be performed in a Biohazard Cabinet to minimize the risk of infection (eg HIV, Hepatitis-B) from potentially infectious blood samples. However, as long as aseptic techniques are used, plasma harvesting can be performed outside of a Biohazard cabinet. 'Safe Laboratory Practices' should be followed, including the use of protective clothing such as gloves, gown, safety eyewear etc, as suggested by relevant regulators.

When harvesting the plasma from above the sedimented red blood cells or gel plug, what volume do I need to remove? Is this important?

As little as 100 μ L of plasma is sufficient, as only 50 μ L of plasma is required to perform the ELISA. (55 μ L is required if a 1:10 dilution is tested well). 200 μ L will leave sufficient plasma for reference (re-testing) purposes, if required. It is generally possible to take greater than 300 μ L. Validation studies have shown that IFN- γ is evenly distributed in the plasma and the volume removed is not critical. The volume of plasma available can vary from patient to patient.

The QuantiFERON[®] -CMV method also allows for direct sampling of plasma from the blood collection tube using an automated system. This allows the plasma harvesting and ELISA to be performed with minimal operator input.

I want to maximise the cost effectiveness of the QuantiFERON[®] -CMV assay by batching my samples. What is the stability associated with the harvested plasma?

Harvested plasma (or plasma stored in the blood collection tubes after centrifugation) can be stored at 2°C to 8°C for up to 28 days, or below -20°C for extended periods; plasma kept at -70°C are less likely to form clots. For short-term storage (less than 28 days), it is better to refrigerate plasma samples rather than freeze them, due to possible fibrin clot formation.

What should I do if "clots" form in my plasma samples during their storage at -20°C?

Upon thawing, frozen plasma samples may require centrifugation to sediment the clots that can form during the freeze/thaw process. A guide to dealing with clotted plasma samples is outlined in the Package Insert.

Do I need to use microtubes for storing harvested plasma? Can I use more "cost effective" ELISA plates in this instance?

Uncoated ELISA plates can be used to store harvested plasma. Regardless of type, all plasma storage vessels should be sealed to avoid evaporation of samples.

Step 3. Human IFN- γ ELISA

The QuantiFERON[®] -CMV assay requires the use of the QuantiFERON[®] -CMI ELISA to measure levels of IFN- γ . The procedure detailed in the QuantiFERON[®] -CMV Package Insert for should be followed instead of the Package Insert for the QuantiFERON[®] -CMI ELISA.

Do I test QuantiFERON -CMV plasma samples neat or diluted?

If quantitative measurements of the IFN- γ (in IU/mL) are required for all samples, then you need to test the CMV and Mitogen plasmas neat as well as diluted 1:10 in Green Diluent. Therefore you need to allow for 5 ELISA wells per patient being tested. The ELISA is accurate for plasma samples containing up to 10 IU/mL. Use the neat plasma values if below 10 IU/mL, otherwise use the values from the diluted samples (multiplied by 10).

What is the stability associated with:

- a) Kit Standard?
- b) Conjugate 100X Concentrate?
- c) Wash Buffer?

- a) Reconstituted IFN- γ Standard may be kept for up to 3 months if stored at 2°C to 8°C (the date of reconstitution should be noted). Reconstituted IFN- γ standard should be equilibrated at Room Temperature (17°C to 27°C) for 1 hour before use.
- b) Once reconstituted, the Conjugate 100X Concentrate must be used within 3 months or discarded. Working strength conjugate (Conjugate 100X Concentrate mixed with Green Diluent) must be used within 6 hours of its preparation. Any unused Conjugate 100X Concentrate must be returned immediately to 2°C to 8°C following its use.
- c) Working strength Wash Buffer may be stored at Room Temperature (17°C to 27°C) for up to 2 weeks.

Can I use the QuantiFERON[®] -CMI ELISA plates immediately after their removal from the fridge?

No. Sealed ELISA plates should be allowed to equilibrate at Room Temperature for at least 1 hour before opening the foil bag.

Do I require an automated Plate Washer?

No. Although an automated plate washer is recommended, manual washing can be performed following the procedure as outlined in the QuantiFERON[®] -CMV Package Insert.

How important is washing during the QuantiFERON® -CMI ELISA?

As with most ELISAs, inadequate or incorrect washing is the single most common cause of QuantiFERON® -CMI ELISA error. If you have any such problems, please check the following:

- If bubbles and froth form during the wash steps, the flow rate of the wash cycle should be adjusted (usually lowered) to prevent this from occurring.
- Wash volumes should allow the wash buffer to reach the top of each well (preferably with a positive meniscus forming over the rim of each well).
- Ensure all wells receive sufficient and equal wash buffer. Blocked washer probes can be cleaned with a fine wire.
- 6 complete washes are suggested as a minimum in the Package Insert, however additional washes can be performed without affecting the performance of the assay.
- A soak period of at least 5 seconds between each cycle is recommended.

My results are not as I had anticipated. What could be the problem?

General ELISA problems with the possible causes and appropriate solutions are listed in the tables below.

Non specific Color Development

POSSIBLE CAUSE	SOLUTION
Incomplete washing of the plate	Wash the plate at least 6 times with 400 µL/well of wash buffer. More than 6 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
Cross-contamination of ELISA wells	Take care pipetting and mixing samples to minimize risk.
Components have expired	Ensure kit is used within the expiry date. Ensure that reconstituted Standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.
Components are contaminated	Avoid contamination of components.

High Background

POSSIBLE CAUSE	SOLUTION
Incomplete washing of the plate_	Wash the plate at least 6 times with 400 µL/well of wash buffer. More than 6 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
Conjugate reconstitution/dilution error_	Conjugate 100X Concentrate should be reconstituted with 300 µL of distilled water. Working strength conjugate is prepared by diluting the Conjugate 100X

	Concentrate 1/100 in Kit Green Diluent as per the Package Insert._
Incubation temperature too high_	Incubation of the ELISA should be performed at Room Temperature, 17°C to 27°C._
Components have expired	Ensure kit is used within the expiry date. Ensure that reconstituted Standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.
Enzyme Substrate is contaminated.	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.

Low Absorbance

POSSIBLE CAUSE	SOLUTION
Standard Dilution Error	Ensure dilutions of the Kit Standard are prepared correctly as per the Package Insert.
Pipetting error	Ensure pipettes deliver correct volume.
Wash Buffer dilution error	Ensure a 1 in 20 dilution of the wash buffer concentrate is performed to prepare the working strength wash buffer.
Incubation Temperature too low	Incubation of the ELISA should be performed at Room Temperature, 17°C to 27°C.
Incubation time too short_	Incubation of the plate with the conjugate, standards and samples should be for 120 +/- 5 minutes. The Enzyme Substrate Solution is incubated on the plate for 30 minutes._
Incorrect Plate Reader filter used_	Plate should be read at 450 nm with a reference filter of between 620 and 650 nm._
Reagents are too cold_	All reagents, with the exception of the Conjugate Concentrate, must be brought to room temperature prior to commencing the assay. This takes approximately one hour._
Kit / Components have expired.	Ensure kit is used within the expiry date. Ensure that reconstituted Standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.

Poor Standard Curve

POSSIBLE CAUSE	SOLUTION
Incomplete washing of the plate	Wash the plate at least 6 times with 400 μ L/well of wash buffer. More than 6 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
Standard Dilution Error_	Ensure dilutions of the Kit Standard are prepared correctly as per the Package Insert._

Duplicate Variability

POSSIBLE CAUSE	SOLUTION
Poor Mixing	Mix reagents thoroughly by inversion or gentle vortexing prior to their addition to the plate.
Incomplete washing of the plate_	Wash the plate at least 6 times. More than 6 washing cycles may be required depending on the washer being used. A soak time between cycles should be used._
Inconsistent pipetting technique or interruption during assay set-up_	Sample and standard addition should be performed in a continuous manner. All reagents should be prepared prior to commencing the assay._

Step 4. Data Analysis

I have very high Nil control values? What may be the problem?

Under most circumstances, the expected IFN- γ concentration range for the Nil control is below 8 IU/mL. If Nil control values are much greater than this, the result may be due to a technical error. In such instances judgement should be used in determining whether to re-test, and if required, it is recommended that both of the subject's plasma samples be re-assayed. If the Nil result remains high, and contamination of the sample plasma is unlikely, the Nil result is valid. A good practice is to check all Nil control values following each test to make sure they fall within, or are close to, the expected range.

Is there an easy way of calculating and interpreting QuantiFERON[®] -CMV test results?

An outline of the Data Analysis and Test Interpretation method for the QuantiFERON[®] -CMV assay is provided in the Package Insert. Calculation of QuantiFERON[®] -CMV results can be performed using a spreadsheet program.

Alternatively, QuantiFERON[®] -CMV Analysis Software is available from Cellestis (download from Technical Info section of website) to analyse assay raw data, and calculate patient QuantiFERON[®] -CMV results.

The QuantiFERON[®] -CMV Analysis Software allows the simple transfer of raw data (ODs) directly from microplate reader software (or from any spreadsheet program). The Software performs:

- Calculation of a Standard Curve
- Quality Control check of the standard replicates and curve
- Calculation of all sample IFN- γ concentrations (IU/mL) from the Standard Curve
- Refer to the 'Interpretation of Results' guidelines outlined in the QuantiFERON[®] -CMV Package Insert for diagnostic interpretation of results.

How do know if a QuantiFERON[®] -CMV result is positive?

A test is considered positive for an IFN- γ response in the CMV Antigen tube if it is significantly above the Nil IFN- γ IU/mL value. A low response to Mitogen (<0.5 IU/mL) indicates an indeterminate result when a blood sample also has a negative response to the CMV antigens. This pattern may occur with insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, incorrect filling/mixing of the Mitogen tube, or inability of the patient's lymphocytes to generate IFN- γ . The Nil sample adjusts for background or non-specific IFN- γ in blood samples. The IFN- γ level of the Nil tube is subtracted from the IFN- γ level for the CMV antigen tube and Mitogen tube.

A cut-off of 0.2 IU/mL has been used in preliminary studies (Walker et al. *Transpl Infect Dis* 2007; DOI: 10.1111/j.1399-3062.2006.00199.x) and the level of agreement between IFN- γ responses to CMV peptides, as measured by QuantiFERON[®] -CMV, and anti-CMV serology information was 97% in healthy individuals. All sero-negative individuals were negative by QuantiFERON[®] -CMV and 94% of seropositive individuals were positive by QuantiFERON[®] -CMV. Similar results were obtained testing samples from solid organ transplantation patients.

How do you convert IU/mL to pg/mL of human IFN- γ ?

The potency of the International Reference Standard for Human IFN- γ is given in IU/mL. Desem and Jones (*Clin Diagn Lab Immunol*, 5, 531-536, 1998) described 40 pg/mL of human IFN- γ as being equivalent to 1 IU/mL.