QuantiFERON®- CMV
Package Insert Σ 2 x 96

The whole blood interferon-gamma test measuring responses to Human Cytomegalovirus peptide antigens

IVD

CE

REF 0350-0201

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Contents

Intended Use

QuantiFERON-CMV (QF-CMV) is an in vitro assay using a peptide cocktail simulating human cytomegalovirus (CMV) proteins to stimulate cells in heparinized whole blood. Detection of interferon-gamma (IFN-γ) by Enzyme-Linked Immunosorbent Assay (ELISA) is used to quantify in vitro responses to these peptide antigens that are associated with immune control of CMV infection. Loss of this immune function may be associated with development of CMV disease. The intended use of QF-CMV is to monitor a patient's level of anti-CMV immunity.

QF-CMV is not a test for determining CMV infection and should not be used to exclude CMV infection.

Introduction

CMV is a herpes virus that infects between 50–85% of adults in the population. It is a frequently occurring complication of immunosuppression, particularly after transplantation, and can significantly contribute to morbidity and mortality in transplant recipients. Current immunosuppressive therapies used to prevent the rejection of a transplanted organ have detrimental effects upon the T-lymphocytes and cell-mediated immune (CMI) responses, resulting in increased susceptibility to viral infections posttransplant. The importance of T-cell function in suppressing CMV replication is also highlighted by the fact that $CD8^+$ CMV-specific cytotoxic T-lymphocytes (CTLs) can protect against virus-associated pathogenesis. The enumeration of $CD8^+$ CMV-specific CTLs in immunosuppressed patients and the production of IFN-γ can be predictive of the risk of developing CMV disease. IFN-γ production can be a functional surrogate for the identification of CMV-specific CTLs.

QF-CMV is an assay for CMI responses to peptide antigens that simulate CMV proteins. The CMV peptides are designed to target $CD8^+$ T cells, including A1, A2, A3, A11, A23, A24, A26, B7, B8, B27, B35, B40, B41, B44, B51, B52, B57, B58, B60 and Cw6 (A30, B13) HLA Class I haplotypes covering >98% of human population. Individuals infected with CMV usually have $CD8^+$ lymphocytes in their blood that recognize these antigens. This recognition process involves the generation and secretion of the cytokine, IFN-γ. The detection and subsequent quantification of IFN-γ forms the basis of this test.

Principles of the Assay

The QF-CMV test is performed in 2 stages. First, whole blood is collected into each of the QF-CMV blood collection tubes, which include a Nil Control tube, CMV Antigen tube, and a Mitogen tube.

The Mitogen tube is used in the QF-CMV test as a positive control. This may be especially warranted where there is doubt as to the individual's immune status.

The tubes should be incubated at 37°C as soon as possible, and within 16 hours of collection. Following a 16 to 24 hour incubation period, the tubes are centrifuged, the plasma is removed and the amount of IFN-γ (IU/ml) measured by QF-CMV ELISA.

The amount of IFN-γ in plasma samples from CMV Antigen and Mitogen tubes may often be above the upper limits of most ELISA readers, even when individuals are moderately immunosuppressed. For **qualitative** results, use the values calculated for neat plasma. For **quantitative** results, where actual IU/ml values are required, plasma samples should be diluted 1/10 in Green Diluent and assayed in the ELISA together with neat plasma.

> **Note:** For samples that are within the range of the QF-CMV ELISA (i.e., up to 10 IU/ml), the result obtained with the neat plasma sample should be used. For such IFN-γ concentrations, values obtained using the 1/10 dilution of the plasma samples may be inexact.

A test is considered reactive for an IFN-γ response when the CMV Antigen tube reads significantly above the Nil IFN-γ IU/ml value. The Mitogen-stimulated plasma sample serves as an IFN-γ positive control for each specimen tested. A low response to Mitogen indicates an indeterminate result when a blood sample also has a non-reactive 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, such as with recent transplant patients. 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 (refer to "Interpretation of Results" on page 15 of this Package Insert for an outline of how QF-CMV results are interpreted).

Time Required for Performing Assay

The time required to perform the QF-CMV assay is estimated below; the time of testing multiple samples when batched is also indicated:

37°C incubation of blood tubes:	16 to 24 hours
ELISA:	Approx. 3 hours for 1 ELISA plate
	Less than 1 hour labor
	Add 10 to 15 minutes for each extra plate

Reagents and Storage

CMV and Control Antigen Blood Collection Tubes (Single Patient Pack)	
Catalog no.	**0192-0301**
Number of preps	**1**
QuantiFERON Nil Control (gray cap)	1 tube
CMV Antigen (blue cap)	1 tube
QuantiFERON Mitogen Control (purple cap)	1 tube
Package Insert	1

QuantiFERON-CMV ELISA Components	
Catalog no.	**0350-0201**
Microplate strips	24 x 8 well strips
Human IFN-γ Standard, lyophilized	1 x vial
Green Diluent	1 x 30 ml
QuantiFERON Conjugate 100X Concentrate, lyophilized	1 x 0.3 ml
QuantiFERON Wash Buffer 20X Concentrate	1 x 100 ml
QuantiFERON Enzyme Substrate Solution	1 x 30 ml
QuantiFERON Enzyme Stopping Solution	1 x 15 ml

Materials Required But Not Provided

- 37°C incubator; CO_2 not required
- Calibrated variable–volume pipets for delivery of 10 µl to 1000 µl with disposable tips
- Calibrated multichannel pipet capable of delivering 50 µl and 100 µl with disposable tips
- Microplate shaker
- Deionized or distilled water, 2 liters
- Microplate washer (automated washer recommended)
- Microplate reader fitted with 450 nm filter and 620 nm to 650 nm reference filter

Storage and Handling

Blood Collection Tubes

- Store blood collection tubes at 4°C to 25°C.
- The shelf life of the QuantiFERON-CMV blood collection tubes is a maximum of 15 months from date of manufacture, when stored at 4°C to 25°C.

ELISA Kit Reagents

- Store kit at 2°C to 8°C.
- Always protect Enzyme Substrate Solution from direct sunlight.

Reconstituted and Unused Reagents

For instructions on how to reconstitute the reagents, please see "Directions for Use — Stage 2" (steps 3 and 5 on pages 11 and 12).

- The reconstituted Kit Standard may be kept for up to 3 months if stored at 2°C to 8°C.

Note the date on which the Kit Standard was reconstituted.

- Once reconstituted, unused QuantiFERON Conjugate 100X Concentrate must be returned to storage at 2°C to 8°C and must be used within 3 months.

Note the date on which the Conjugate was reconstituted.

- Working strength conjugate must be used within 6 hours of preparation.
- Working strength Wash Buffer may be stored at room temperature (17°C to 27°C) for up to 2 weeks.

Warnings and Precautions

For In Vitro Diagnostic Use.

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please ccnsult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

CAUTION: Handle human blood as if potentially infectious. Observe relevant blood handling guidelines.

The following risk and safety phrases apply to components of the QF-CMV ELISA Kit.

QuantiFERON Enzyme Stopping Solution

Contains sulfuric acid: Irritant. Risk and safety phrases:* R36/38, S26-36/37/39

- **Green Diluent** contains normal mouse serum and casein, which may trigger allergic responses; avoid contact with skin.

For Chemical Emergency

Spill, Leak, Exposure, or Accident

Call CHEMTREC Day or Night

Within USA and Canada: 1-800-424-9300

Outside USA and Canada: +1-703-527-3887 (collect calls accepted)

Further information

Safety Data Sheets: www.qiagen.com/safety

* R36/38: Irritating to eyes and skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection.

Specimen Collection and Handling

Important points before starting:

Deviations from the QF-CMV package insert may yield erroneous results. Please read the instructions carefully before use.

- Do not use kit if any reagent bottle shows signs of damage or leakage prior to use.
- Do not mix or use ELISA reagents from other QF-CMV ELISA kit batches.
- Discard unused reagents and biological samples in accordance with Local, State, and Federal regulations.
- Do not use the QF-CMV blood collection tubes or QF-CMV ELISA kits after the expiration date.

QF-CMV uses the following blood collection tubes:

1. Nil Control (gray cap)
2. CMV Antigen (blue cap)
3. Mitogen Control (purple cap)

Antigens have been dried onto the inner wall of the blood collection tubes, so it is essential that the contents of the tubes be thoroughly mixed with the blood. The tubes must be transferred to a 37°C incubator as soon as possible and within 16 hours of collection.

The following procedures should be followed for optimal results:

1. **For each patient collect 1 ml of blood by venipuncture directly into each of the QF-CMV blood collection tubes.**
   - As 1 ml tubes draw blood relatively slowly, keep the tube on the needle for 2–3 seconds once the tube appears to have completed filling, to ensure that the correct volume is drawn.

     The black mark on the side of the tubes indicates the 1 ml fill volume. QF-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 line, it is recommended to obtain another blood sample.
   - QF-CMV blood collection tubes have been validated to draw between 0.8 ml and 1.2 ml at altitudes from sea level to 810 meters (2650 feet). Above this altitude users should ensure that blood is drawn into each tube within these limits. If low blood draw does occur, blood can be collected using a syringe and 1 ml transferred to each of the 3 tubes. For safety reasons, this is best performed by removing the syringe needle, ensuring appropriate safety procedures, removing the caps from the three QF-CMV tubes and adding 1 ml of blood to each (to the black mark on the side of the tube label). Replace the tubes caps securely and mix as described below.
   - If a butterfly needle is being used to collect blood, a "purge" tube should be used to ensure that the tubing is filled with blood prior to the QF-CMV blood collection tubes being used.
2. **Immediately after filling the tubes, shake them ten (10) times just firmly enough to ensure that the entire inner surface of the tube is coated with blood, to dissolve antigens on tube walls.**
   - Tubes should be kept between 17°C–25°C at the time of filling.
   - Over-energetic shaking may cause gel disruption and could lead to aberrant results.
3. **Label tubes appropriately.**
4. **The tubes must be transferred to a 37°C ± 1°C incubator as soon as possible, and within 16 hours of collection. Do not refrigerate or freeze the blood samples.**

Directions for Use

Stage 1 — Incubation of blood and harvesting of plasma

1. **If the blood is not incubated immediately after collection, mixing of the tubes must be repeated immediately prior to incubation, as described in Step 2 of previous section.**
2. **Incubate the tubes UPRIGHT at 37°C for 16 to 24 hours. The incubator does not require CO_2 or humidification.**
3. **Following incubation, blood collection tubes may be held between 2°C and 27°C for up to 3 days prior to the next step. After incubation of the tubes at 37°C, centrifuge the tubes for 15 minutes at 2000 to 3000 RCF (g). The gel plug will separate the cells from the plasma. If this does not occur, the tubes should be re-centrifuged at a higher speed.**
   - It is possible to harvest the plasma without centrifugation, but additional care is required to remove the plasma without disturbing the cells.
4. **After centrifugation, avoid pipetting up and down or mixing plasma by any means prior to harvesting. At all times, take care not to disturb material on the surface of the gel.**
   - Plasma samples should only be harvested using a pipet.
   - Plasma samples can be loaded directly from centrifuged blood collection tubes into the QF-CMV ELISA plate, including when automated ELISA workstations are used.
   - Plasma samples can be stored for up to 28 days at 2°C to 8°C or, if harvested, or below –20°C (preferably less than –70°C) for extended periods in tubes or plasma storage containers.

Stage 2 — QuantiFERON-CMV ELISA for Human IFN-γ

1. **All plasma samples and reagents, except for Conjugate 100X Concentrate, must be brought to room temperature (17°C to 27°C) before use. Allow at least 60 minutes for equilibration.**
2. **Remove strips that are not required from the frame, reseal in the foil pouch, and return to the refrigerator for storage until required.**

   Allow at least one strip for the QF-CMV ELISA Standards and sufficient strips for the number of patients being tested. After use, retain frame and lid for use with remaining strips.
3. **Reconstitute the freeze dried Kit Standard with the volume of deionized or distilled water indicated on the label of the Standard vial. Mix gently to minimize frothing and ensure complete resolubilization. Reconstitution of the Standard to the stated volume will produce a solution with a concentration of 8.0 IU/ml.**
4. **The Standard Curve is prepared using 3 dilutions of the Kit Standard and Green Diluent alone as Standard 4 (0 IU/ml).**

   Use the reconstituted Kit Standard to produce a dilution series of 3 IFN-γ concentrations. Dilute in Kit Green Diluent (GD) (see Figure 1 on next page). The standards should be assayed at least in duplicate; the following steps generate sufficient volume for this.

   a. Label 4 tubes "S1", "S2", "S3", "S4".

   b. Add 150 µl of Green Diluent to 4 tubes (S1–S4).

   c. Add 150 µl of the Kit Standard to S1 and mix thoroughly.

   d. Transfer 50 µl from S1 to S2 and mix thoroughly.

   e. Transfer 50 µl from S2 to S3 and mix thoroughly.

f. Green Diluent alone serves as the zero standard (S4).

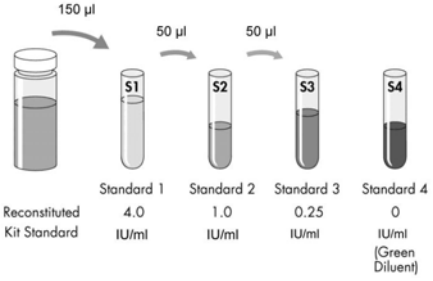

Figure 1. Preparation of Standard Curve. Prepare fresh dilutions of the Kit Standard for each ELISA session.

5. **Reconstitute freeze dried QuantiFERON Conjugate 100X Concentrate with 0.3 ml of deionized or distilled water. Mix gently to minimize frothing and ensure complete solubilization of the Conjugate.**
6. **Working strength conjugate is prepared by diluting the required amount of reconstituted Conjugate 100X Concentrate in Green Diluent as set out in Table 1 – Conjugate Preparation.**
   - Mix thoroughly but gently to avoid frothing.
   - Return any unused Conjugate 100X Concentrate to 2°C to 8°C immediately after use.
   - Use only Green Diluent.

Table 1. Conjugate preparation

Number of strips	Volume of Conjugate 100X Concentrate	Volume of Green Diluent
2	10 µl	1.0 ml
3	15 µl	1.5 ml
4	20 µl	2.0 ml
5	25 µl	2.5 ml
6	30 µl	3.0 ml
7	35 µl	3.5 ml
8	40 µl	4.0 ml
9	45 µl	4.5 ml
10	50 µl	5.0 ml
11	55 µl	5.5 ml
12	60 µl	6.0 ml

7. **Prior to assay, plasmas should be mixed to ensure that IFN-γ is evenly distributed throughout each sample. Also dilute CMV and Mitogen plasmas 1/10 in Green Diluent (10 µl plasma mixed with 90 µl GD) if quantitative results are required. The Nil plasma should not be diluted.**

   It is recommended that the following samples are tested:

   - Nil, CMV Antigen, Mitogen, CMV Antigen (1/10), Mitogen (1/10)

   However, the following patient sample options are also supported by the QuantiFERON-CMV Analysis Software:

   - Nil, CMV Antigen, Mitogen
   - Nil, CMV Antigen (1/10), Mitogen (1/10)
   - Nil, CMV Antigen, Mitogen, CMV Antigen (1/10)
   - Nil, CMV Antigen (1/10), Mitogen

8. **Add 50 µl of freshly prepared working strength conjugate to the required ELISA wells using a multichannel pipet.**
9. **Add 50 µl of test plasma samples to appropriate wells using a multichannel pipet. Finally, add 50 µl each of the Standards 1 to 4.**
10. **Mix the conjugate and plasma samples/standards thoroughly using a microplate shaker for 1 minute.**
11. **Cover each plate with a lid and incubate at room temperature (17°C to 27°C) for 120 ± 5 minutes.**
    - Plates should not be exposed to direct sunlight during incubation.
12. **During the incubation, dilute one part Wash Buffer 20X Concentrate with 19 parts deionized or distilled water and mix thoroughly. Sufficient Wash Buffer 20X Concentrate has been provided to prepare 2 liters of working strength wash buffer.**

    Wash wells with **400 µl** of working strength wash buffer for at least 6 cycles. An automated plate washer is recommended.

    - Thorough washing is very important to the performance of the assay. Ensure each well is **completely filled** with wash buffer to the top of the well for each wash cycle. A soak period of at least 5 seconds between each cycle is recommended.
    - Standard laboratory disinfectant should be added to the effluent reservoir, and established procedures followed for the decontamination of potentially infectious material.
13. **Tap plates face down on absorbent towel to remove residual wash buffer. Add 100 µl of Enzyme Substrate Solution to each well and mix thoroughly using a microplate shaker.**
14. **Cover each plate with a lid and incubate at room temperature (17°C to 27°C) for 30 minutes.**
    - Plates should not be exposed to direct sunlight during incubation.
15. **Following the 30-minute incubation, add 50 µl of Enzyme Stopping Solution to each well and mix.**
    - Enzyme Stopping Solution should be added to wells in the same order and at approximately the same speed as the substrate in step 13.
16. **Measure the Optical Density (OD) of each well within 5 minutes of stopping the reaction using a microplate reader fitted with a 450 nm filter and with a 620 nm to 650 nm reference filter. OD values are used to calculate results.**

Calculations and Test Interpretation

QuantiFERON-CMV Analysis Software, for the analysis of raw data and calculation of results, is available from QIAGEN at www.QuantiFERON.com.

The software performs a quality control assessment of the assay, generates a standard curve and provides a test result for each patient, as detailed in the Interpretation of Results section.

As an alternative to using the QF-CMV Analysis Software, results can be determined according to the following method.

Generation of Standard Curve

Determine the mean OD values of the Kit Standard replicates on each plate.

Construct a $\log_{(e)}$-$\log_{(e)}$ standard curve by plotting the $\log_{(e)}$ of the mean OD (y-axis) against the $\log_{(e)}$ of the IFN-γ concentration of the standards in IU/ml (x-axis), omitting the zero standard from these calculations. Calculate the line of best fit for the standard curve by regression analysis.

Use the standard curve to determine the IFN-γ concentration (IU/ml) for each of the test plasma samples, using the OD value of each sample.

These calculations can be performed using software packages available with microplate readers, and standard spreadsheet or statistical software (such as Microsoft® Excel®). It is recommended that these packages be used to calculate the regression analysis, the coefficient of variation (%CV) for the standards, and the correlation coefficient (r) of the standard curve.

Quality Control of Test

The accuracy of test results is dependent on the generation of an accurate standard curve. Therefore, results derived from the standards must be examined before test sample results can be interpreted.

For the ELISA to be valid:

- The mean OD value for Standard 1 must be ≥ 0.600.
- The %CV for Standard 1 and Standard 2 replicate OD values must be < 15%.
- Replicate OD values for Standards 3 and 4 must not vary by more than 0.040 optical density units from their mean.
- The correlation coefficient (r) calculated from the mean absorbance values of the standards must be ≥ 0.98.

If the above criteria are not met, the run is invalid and must be repeated.

The mean OD value for the Zero Standard (Green Diluent) should be ≤ 0.150. If the mean OD value is > 0.150 the plate washing procedure should be investigated.

Interpretation of Results

QuantiFERON-CMV results are interpreted using the following criteria:

CMV minus Nil (IU/ml)*	Mitogen minus Nil (IU/ml)	QF-CMV result	Report/Interpretation
< 0.2	≥ 0.5	Non-reactive	Anti-CMV immunity NOT detected
≥ 0.2	Any	Reactive	Anti-CMV immunity detected
< 0.2	< 0.5	Indeterminate†	Result indeterminate for CMV responsiveness

* IFN-γ responses to the CMV Antigen and Mitogen positive control can be commonly outside the range of the microplate reader. This has no impact on qualitative results.

† Refer to Troubleshooting section for possible causes.

Limitations

Results of QuantiFERON-CMV testing must be used in conjunction with each individual's epidemiological history, current medical status, and other diagnostic evaluations.

Unreliable or indeterminate results may occur due to:

- Deviation from the procedure described in the Package Insert.
- Excessive levels of IFN-γ in Nil tube.
- Longer than 16 hours from blood specimen drawing to incubation at 37°C.

Expected Values

Expected IFN-γ values using QuantiFERON-CMV were obtained from testing 591 samples from healthy adults, 341 of whom were CMV seropositive and 250 were seronegative. In the 250 healthy adult subjects without CMV infection, as determined by anti-CMV serology (CMV seronegative), 100% of subjects produced IFN-γ responses of < 0.2 IU/ml to the CMV Antigen tube (minus Nil). The distribution of CMV Antigen tube (minus Nil) for the 341 healthy subjects with CMV infection, as determined by anti-CMV serology (CMV seropositive), are shown in Figure 1.

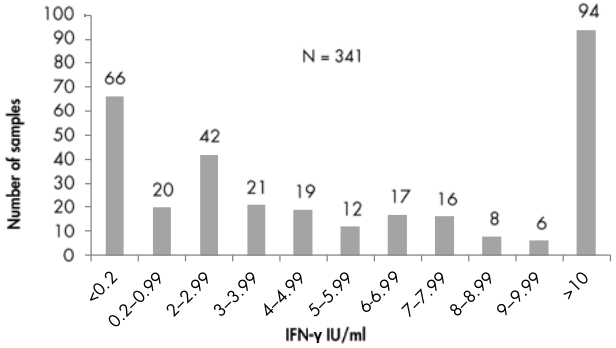

Figure 1. Distribution of CMV-Nil IFN-γ responses in seropositive healthy subjects (n=341).

The distribution of Mitogen (minus Nil background) results in 731 normal blood samples from healthy adult subjects, irrespective of known CMV infection is shown in Figure 2. Mitogen (minus Nil) result of less than 0.5 IU/ml indicates either test failure or that the person is in an immunocompromised state. In a healthy population, only 2/731 results fell into this category.

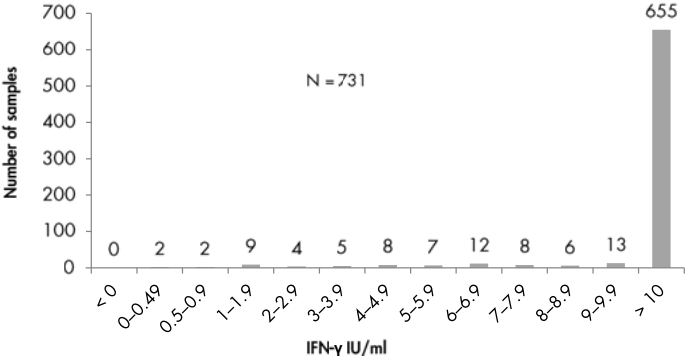

Figure 2. Distribution of Mitogen-Nil IFN-γ responses in healthy adult subjects (n = 731).

Expected values for Nil tubes are shown in Figure 3. Data is derived from 1020 plasma samples from healthy adult subjects tested using the QuantiFERON-CMV ELISA.

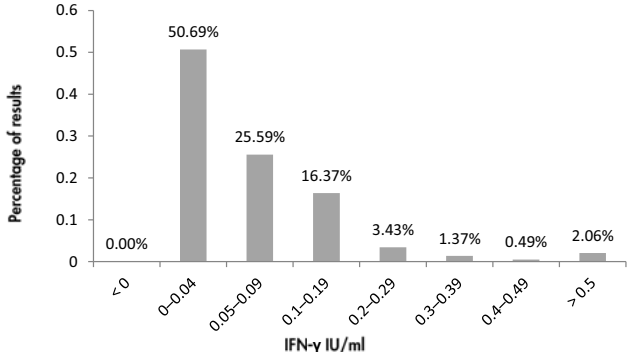

Figure 3. Distribution (expressed as % of population) of Nil IFN-γ responses in healthy adult subjects (n = 1020).

Performance Characteristics

Predicate Testing

A test threshold for detecting prior CMV exposure using QF-CMV was established following the analysis of results from a group of healthy subjects (n = 223) whereby QF-CMV results were compared to CMV serological results. A ROC analysis determined that a test threshold of 0.04 IU/ml (after nil subtraction) provided optimal positive and negative predictive values for QF-CMV (area under the curve = 0.9679 [95%CI = 0.9442 to 0.9915, $p < 0.0001$]), and thus represented the threshold at which this assay performed its intended use most effectively in a healthy population.

In predicate testing, QF-CMV's performance was compared to the SeraQuest CMV IgG serology test (Quest International). The QF-CMV assay showed 95% (294/310 individuals) agreement with the predicate anti-HCMV serology test in healthy individuals, with none of the 149 seronegative donors showing any reactivity by QF-CMV, and 145 of 161 seropositive donors demonstrating a reactive IFN-γ response. Overall positive agreement was 90% with a negative agreement value of 100%. The level of agreement between IFN-γ responses to CMV peptides, as measured by QF-CMV, in healthy volunteers, and the anti-CMV serology status of these subjects using the SeraQuest CMV IgG serology test are shown in Table 2.

Table 2. Agreement between QuantiFERON-CMV and CMV IgG serology test in healthy subjects.

		CMV Serology		
		Positive	Negative	Total
QuantiFERON-CMV	Reactive	145	0	145 (46.8%)
	Non-reactive	16	149	165 (53.2%)
	Total	161 (51.9%)	149 (48.1%)	310 (100%)

Assay threshold

The recommended clinical test threshold for this assay is 0.2 IU/ml in the CMV Antigen tube (minus Nil) although different thresholds may be validated for different clinical settings. The reasoning lies in the fundamental immunological differences between a normal test population and populations in which the test is considered clinically useful — specifically immunosuppressed persons who due to immunosuppression are at risk of developing symptomatic CMV infection and/or disease. In such high-risk individuals, QF-CMV's clinical utility lies in accurately detecting the level of anti-CMV immunity in these subjects, as the lack of immunity may be associated with development of CMV disease (1–5, 7, 8, 11–16).

Clinical Studies

As there is no definitive standard for confirming or excluding the diagnosis of cytomegalovirus infection, an estimate of sensitivity and specificity for QF-CMV cannot be practically evaluated. The specificity and sensitivity of QF-CMV was approximated by evaluating the level of agreement between IFN-γ responses to CMV peptides, as measured by QF-CMV in healthy volunteers and the anti-CMV serology status of these subjects using a CMV IgG serology test.

Specificity of QF-CMV was approximated by evaluating false-positive rates (QF-CMV Reactive response) in healthy volunteers with no evidence of prior CMV exposure (CMV seronegative individuals). Sensitivity was approximated by evaluating healthy volunteers with evidence of prior CMV exposure (CMV seropositive individuals). Although QF-CMV utilizes a large number of CMV specific epitopes from different CMV proteins, thus providing broad clinical application to a wide range of the population with diverse HLA Class I haplotypes, the coverage of these peptides is not 100%. As the HLA haplotypes of subjects tested against CMV serology were unknown, a small percentage of serology positive individuals were expected to be nonresponsive to the QF-CMV tubes.

Specificity

In a study conducted in healthy subjects with no evidence of prior CMV exposure (CMV seronegative individuals where n = 250), the level of agreement between IFN-γ responses to CMV peptides, as measured by QF-CMV, and anti-CMV serology information was found to be 100%.

In all other specificity evaluations conducted in recipients of solid organ transplants (1, 3, 4, 8, 12, 14–16), recipients of hematopoietic stem cell transplants (7, 13) and HIV-infected patients (2), the level of agreement

between IFN-γ responses to CMV peptides, as measured by QF-CMV and anti-CMV serology, has consistently been demonstrated at 100%.

Sensitivity

In a study conducted in healthy subjects with evidence of prior CMV exposure (CMV seropositive individuals where n = 341), the level of agreement between IFN-γ responses to CMV peptides as measured by QF-CMV, and anti-CMV serology was found to be 80.6% (275/341). The observed discordance may be due to the use of the higher test threshold (0.2 IU/ml), false-positive CMV serology, or the subjects' nonresponsiveness to the CMV peptides included in the assay.

In sensitivity evaluations conducted in solid organ transplant recipients (1, 3, 4, 8, 12, 14–16), hematopoietic stem cell transplant recipients (7, 13) and HIV-infected patients (2), some slightly lower levels of agreement have been found between IFN-γ responses to CMV peptides, as measured by QF-CMV, and CMV seropositive responses in these patients. The lower level of agreement may be a result of either false-positive CMV serology, the patients' nonresponsiveness to the CMV peptides included in the assay, or the absence of reactive T cells in these patients due to their immunosuppression.

Studies highlighting clinical utility

Both serology and QF-CMV describe their intended use as allowing the detection of immunity to CMV. Within a transplant setting, CMV serology is widely used pretransplantation to establish risk of CMV complications occurring in the recipient posttransplantation, but has limited value itself posttransplantation. Alternatively, QF-CMV may be used in transplant recipients to assess the level of CMV immunity in those patients at risk of developing symptomatic CMV infection and/or disease due to immunosuppression (6, 9–11).

A number of published clinical studies in a variety of transplant cohorts have now demonstrated the utility of QuantiFERON-CMV (1–5, 7, 8, 11–16).

In a large study of 108 solid organ transplant recipients (4), patients with a QF-CMV Reactive result at the completion of anti-CMV prophylaxis had a significantly lower rate of late onset disease compared with those having a QF-CMV Non-reactive result (5.3% vs. 22.9% respectively, p=0.044) (Figure 4).

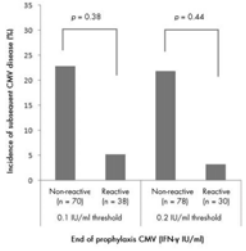

Figure 4. Rates of late onset CMV disease in patients with a QuantiFERON-CMV Reactive result vs. a QuantiFERON-CMV Non-reactive result at the end of prophylaxis. Data reproduced from Kumar et al.(4)

Furthermore, patients with a reactive QF-CMV test upon completion of prophylaxis remained free from CMV disease more often, and for longer (Figure 5), indicating that QF-CMV may be used to identify those at risk of developing late-onset CMV disease.

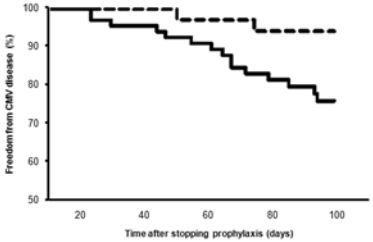

Figure 5. Time to development of CMV disease in patients with a QuantiFERON-CMV Reactive result (highlighted by broken line) vs. a QuantiFERON-CMV Non-reactive result (highlighted by solid line) at the end of prophylaxis. Data reproduced from Kumar et al.(4)

This study also highlighted that in the cohort of transplantation patients at highest risk of developing CMV disease (CMV seronegative transplantation recipients who receive an organ from a CMV seropositive donor, i.e., D+/R-) a QF-CMV Reactive result any time post-prophylaxis was associated with a 90% chance of remaining free from CMV disease.

In a study of 37 solid organ transplant patients (12), the assessment of CMV specific CD8+ T-cell responses by QF-CMV assisted the prediction of spontaneous viral clearance compared to CMV disease progression, following elevations in CMV viremia. In this study, 24/26 patients (92.3%) with a QF-CMV Reactive result, spontaneously cleared CMV virus while only 5/11 (45.5%) patients with a QF-CMV Non-reactive result had the same outcome.

A study of 67 Lung Transplant recipients assessing CMV viremia episodes posttransplant (14), observed that 18/25 (72%) CMV viremia episodes were preceded by a Non-reactive QF-CMV result, versus 4/16 (25%) episodes that were preceded by a Reactive QF-CMV response (Fisher's exact test, $p = 0.0046$, see Figure 6).

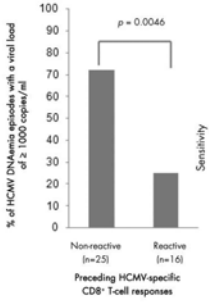

Figure 6. Statistical analysis of CMV-specific CMV specific CD8+ T- cell responses as detected by QuantiFERON-CMV and the development of CMV viremia (Fisher's exact test, $p = 0.0046$). Data reproduced from Weseslindtner et al (14).

In a large multicenter prospective study of 127 D+/R- solid organ transplant recipients (15), all of whom received antiviral prophylaxis, patients with a QF-CMV Reactive result (using a 0.1 IU/ml test threshold) at any timepoint

following the completion of anti-CMV prophylaxis had a significantly lower rate of late onset disease at 12 months posttransplant, compared with those having a QF-CMV Non-reactive result and an Indeterminate result (6.4% vs. 22.2% vs. 58.3%, respectively, p<0.001). When classifying indeterminate results as also being "Non-reactive", the incidence of subsequent CMV disease was 6.4% vs. 26.8%, p = 0.024 (see Figure 7). The positive and negative predictive values of QF-CMV for protection from CMV disease were 0.90 (95% CI 0.74-0.98) and 0.27 (95% CI 0.18-0.37) respectively, indicating that a QuantiFERON-CMV Reactive result any time post-prophylaxis was associated with a 90% chance of remaining free from CMV disease. This study found that QF-CMV may be useful to predict if patients are at low, intermediate, or high risk for the development of subsequent CMV disease after prophylaxis.

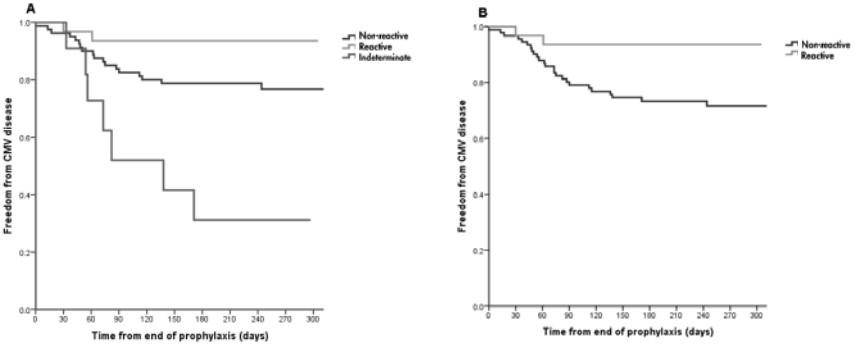

Figure 7. Kaplan-Meier curves of the incidence of CMV disease according to the result of the QF-CMV assay. **A** Reactive vs. Non-reactive vs. Indeterminate QF-CMV results (log rank test, $p<0.001$). **B** Reactive vs. Non-reactive, where indeterminate results were considered as being "Non-reactive" (log rank test, $p = 0.024$).

In a prospective study of 55 solid organ transplant recipients (16) where the relationship between pretransplant QF-CMV results and posttransplant CMV replication episodes were analyzed, it was found that a higher incidence of posttransplant CMV replication was observed in R(+) recipients with a pretransplant QF-CMV Non-reactive result (7/14 or 50%), compared to those R(+) recipients with a QF-CMV Reactive result (4/30 or 13.3%).

This study found that pretransplant QF-CMV Non-reactive recipients who received an organ from a CMV-seropositive donor had a tenfold increased risk of CMV replication compared to those pretransplant QF-CMV reactive recipients (adjusted OR 10.49, 95% CI 1.88–58.46), and that a pretransplant QF-CMV assay may be useful in predicting the risk for CMV replication after transplantation and thus allow the individualization of CMV infection management after solid organ transplantation.

A number of other studies investigating the detection of CMV-specific $CD8^+$ T-cell responses by QF-CMV in a cohort of transplant recipients have been completed (1, 3, 5, 7, 8, 11, 13) or are currently in progress worldwide.

International consensus guidelines on the management of cytomegalovirus in solid organ transplant

The importance of CMV-specific immune monitoring has been recognized and published in the "International consensus guidelines on the management of cytomegalovirus in solid organ transplantation" (6). These international guidelines, developed by a panel of experts on CMV and solid organ transplant, convened by The Infectious Diseases Section of The Transplantation Society, represent evidence and expert opinion-based consensus guidelines on CMV management including: diagnostics, immunology, prevention, and treatment.

These guidelines concluded that "Immune monitoring of CMV-specific T-cell responses may predict individuals at risk of CMV disease posttransplant and may be useful in guiding prophylaxis and pre-emptive therapies" (6).

Futhermore, the guidelines also provided recommendations as to the attributes of the ideal immune monitoring assay, which included:

- Ability to assess the quantity and function of a transplant recipient's $CD4^+$ and $CD8^+$ T cells
- Ability to measure IFN-γ
- Simple to perform, cost-effective, and reproducibility
- Rapid turnaround time
- Ease of shipping specimens to specialized referral laboratories

QF-CMV meets virtually all the criteria specified by these guidelines, and represents the only standardized, immune monitoring assay capable of detecting IFN-γ, specific for CMV.

Assay Performance Characteristics

The method for measuring IFN-γ concentration by the QF-CMV ELISA has been demonstrated to be linear from zero to 10 IU/ml (Figure 8). The linearity study was performed by placing 5 replicates of 11 plasma pools of known IFN-γ concentrations randomly on the ELISA plate.

The QF-CMV ELISA shows no evidence of a high-dose hook (prozone) effect with concentrations of IFN-γ of up to 100,000 IU/ml.

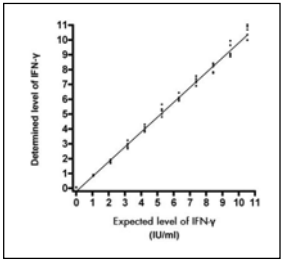

Figure 8. Linearity profile of QF-CMV ELISA determined from testing 5 replicates of 11 plasma samples of known IFN-γ concentrations. The linear regression line has a slope of 1.002 ± 0.011 and a correlation coefficient of 0.99.

Intra- and inter-assay imprecision (%CV) of the QF-CMV ELISA was estimated by testing 20 plasma samples with varying IFN-γ concentrations in replicates of 3, in 3 laboratories, on 3 non-consecutive days, by 3 operators. Thus each sample was tested 27 times, in 9 independent assay runs. One sample was a Nil control and had a

calculated IFN-γ concentration of 0.08 (95% CI 0.07 – 0.09) IU/ml. Of the remaining 19 plasma samples, the range of concentrations was 0.33 (0.31 – 0.34) to 7.7 IU/ml (7.48 – 7.92).

Within run or intra-assay imprecision was estimated by averaging the %CVs for each test plasma containing IFN-γ from each plate run (n=9) and ranged from 4.1 to 9.1%CV. The average within run %CV (±95% CI) was 6.6% ± 0.6%. The zero IFN-γ plasma averaged 14.1%CV.

Total or inter-assay imprecision was determined by comparing the 27 calculated concentrations of IFN-γ for each plasma sample and ranged from 6.6 to 12.3%CV. The overall average %CV (±95% CI) was 8.7% ± 0.7%. The zero IFN-γ plasma showed a 26.1%CV. This level of variation is to be expected because the calculated concentration of IFN-γ is low and variation around a low estimate of concentration will be larger than that for higher concentrations.

The precision profile for QF-CMV ELISA is shown in Figure 9 and indicates that imprecision does not increase with higher concentrations of IFN-γ.

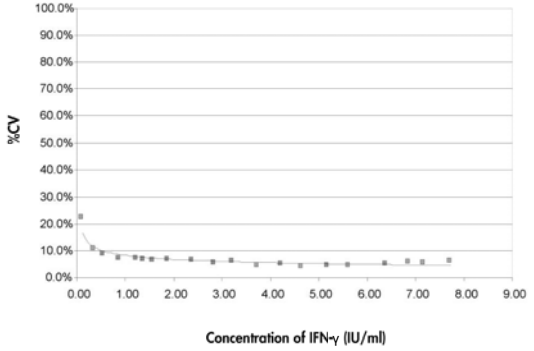

Figure 9. Precision profile of QF-CMV ELISA determined from testing 20 plasma samples in triplicate, on 3 non-consecutive days, at 3 laboratories and by 3 operators. The trend line is a calculation of least squares fit.

A study was conducted to establish the reproducibility of the QF-CMV test using blood samples from 8 subjects with unknown CMV status. Blood for each subject was collected into three sets of QF-CMV tubes (3 x Nil, 3x CMV, and 3x Mitogen). The three sets of tubes were then incubated at three different sites (one set of Nil, CMV, and Mitogen per site), as outlined in the Package Insert. After 16–24 hours incubation, the tubes were centrifuged and the plasma harvested.

ELISAs were subsequently performed three times at each of the three sites, generating three QF-CMV results for each subject per site (9 results total across all sites). Each site employed a different operator. Plates used for the study were not necessarily from within the same lot number, but they were all within their respective expiry dates.

Reproducibility, in terms of both diagnostic status (Reactive, Non-reactive, or Indeterminate) and numerical value, was determined for each blood sample. The reproducibility of the numerical value was only assessed in reactive samples (expressed as %CV), as IFN-γ levels in "Non-reactive" samples were too small to provide any meaningful estimate of precision.

Overall, diagnostic reproducibility was 100% where the QF-CMV diagnostic status of all 8 volunteers was reproduced at all sites on all occasions, with no Indeterminate samples being reported. Reproducibility of Reactive

samples was acceptable both within site and between sites. The mean %CV for each of the test sites was 4.5% (Site 1), 5.9% (Site 2), and 7.3% (Site 3). Overall, the between site %CV was 5.9% for all 5 reactive samples. Percentage coefficient of variance values below 10% are considered excellent.

Technical Information

Indeterminate Results

Indeterminate results may be related to the immune status of the individual being tested, but may also be related to a number of technical factors:

- Longer than 16 hours from blood draw to incubation at 37°C.
- Storage of blood outside the recommended temperature range (17°C to 27°C).
- Insufficient mixing of blood collection tubes.

If technical issues are suspected with the collection or handling of blood samples, repeat the entire QF-CMV test with new blood specimens. Repeating the ELISA testing of stimulated plasmas can be performed if any procedural deviation with the ELISA test is suspected. Indeterminate results (from low Mitogen values) would not be expected to change on repeat unless there was an error with the ELISA testing.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Technical Information provided at: www.QuantiFERON.com. For contact information, see page 26 and the back cover.

ELISA troubleshooting

Low optical density readings for standards

Possible cause	Solution
a) Standard dilution error	Ensure dilutions of the Kit Standard are prepared correctly as per the Package Insert.
b) Pipetting error	Ensure pipets are calibrated and used according to manufacturer's instructions.
c) Incubation temperature too low	Incubation of ELISA should be performed at room temperature (17°C to 27°C).
d) 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.
e) Incorrect plate reader filter used	Plate should be read at 450 nm with a reference filter between 620 and 650 nm.
f) Reagents are too cold	All reagents, with the exception of the Conjugate 100X Concentrate, must be brought to room temperature prior to commencing the assay. This takes approximately 1 hour.
g) Kit/components have expired	Ensure that the kit is used before the expiry date. Ensure reconstituted Standard and Conjugate 100X Concentrate are used within 3 months of the reconstitution date.

Non-specific color development / high background

Possible cause	Solution
a) 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.
b) Incubation temperature too high	Incubation of the ELISA should be performed at room temperature (17°C to 27°C).
c) Kit/components have expired	Ensure that the kit is used before the expiry date. Ensure reconstituted Standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.
d) Enzyme Substrate Solution is contaminated	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.
e) Mixing of plasma in centrifuge tubes before harvesting	Ensure that plasma samples are carefully harvested from above gel without pipetting up and down, taking care not to disturb material on the surface of the gel.

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Abbreviated Test Procedure

Stage 1 — blood incubation

1. Collect patient blood into blood collection tubes and mix by shaking them ten (10) times just firmly enough to ensure that the entire inner surface of the tube has been coated with blood, to dissolve antigens on tube walls.

2. Incubate tubes upright at 37°C ± 1°C for 16 to 24 hours.

3. Following incubation, centrifuge tubes for 15 minutes at 2000 to 3000 RCF (g) to separate the plasma and the red cells.

4. After centrifugation, avoid pipetting up and down or mixing the plasma by any means prior to harvesting. At all times, take care not to disturb the material on the surface of the gel.

Stage 2 — IFN–γ ELISA

1. Equilibrate ELISA components, with the exception of the Conjugate 100X Concentrate, to room temperature for at least 60 minutes.

2. Reconstitute the Kit Standard to 8.0 IU/ml with distilled or deionized water. Prepare four (4) standard dilutions.

3. Reconstitute freeze-dried Conjugate 100X Concentrate with distilled or deionized water.

4. Prepare working strength conjugate in Green Diluent and add 50 µl to all wells.

5. Add 50 µl of test plasma samples and 50 µl standards to appropriate wells. Mix using shaker.

6. Incubate for 120 minutes at room temperature.

7. Wash wells at least 6 times with 400 µl/well of Wash Buffer.

8. Add 100 µl Enzyme Substrate Solution to wells. Mix using shaker.

9. Incubate for 30 minutes at room temperature.

10. Add 50 µl Enzyme Stopping Solution to all wells. Mix using shaker.

11. Read results at 450 nm with a 620 to 650 nm reference filter.

12. Analyze results.

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