

CMV monitoring: the importance of validated flexibility

PCR is the recommended method of monitoring cytomegalovirus in immunocompromised patients. The PCR kit itself is however only part of an overall assay process which also includes sample preparation methods, PCR instrumentation and data analysis modules. This article describes a CMV RT-PCR test system which is flexible enough to be used with various other modules in a fully validated complete assay system.

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Cytomegalovirus related disease is one of the most severe complications of stem cell transplantation. The virus is found in more than 70% of the European adult population, normally residing in a dormant phase in the white blood cells. In immunocompromised patients however the full pathogenic potential of the virus can be unleashed, giving rise to serious pathological conditions. Unless prophylactic or pre-emptive treatment is given, CMV can thus be responsible for many transplantation-related complications which are associated with high mortality.

Prophylactic treatment is however not always the best option to avoid CMV related disease and possible death. Even though prophylactic drug treatment may delay CMV reactivation it cannot eliminate eventual problems. CMV-specific immunity is T-cell mediated and so for the patient to regain immunity after stem cell transplantation the virus has to be reactivated [1]. In addition, prophylactic drugs can be associated with severe adverse effects and can also increase the risk of the development of resistant strains. A pre-emptive monitoring strategy is therefore to be preferred. In this, once virus is detected an appropriate treatment regimen is immediately initiated, before the onset of symptoms. In practice, for pre-emptive strategies to be effective, it is therefore vital to have a sensitive and specific viral detection system.

CMV load is significantly higher in patients who develop disease than in those who do not; the risk of disease development is proportional to the CMV load [2]. Such findings further underline the importance of quantitative viral monitoring in order to determine the appropriate treatment for the patient.

Demands on the monitoring process

In real-time PCR-based diagnosis of CMV, there is unfortunately no consensus among

clinicians as to the preferred sample matrix, sample preparation method or PCR system. A commercially successful CMV monitoring PCR kit must therefore be flexible enough to be able to handle different matrices, and to accommodate different sample extraction methods as well as different real-time PCR systems.

Sample matrices

Different laboratories use different sample matrices for CMV testing. Such matrices include whole blood, blood cells or plasma and the results can sometimes vary significantly from one matrix to the other [3]. It is thus important that the PCR kit be validated for use in both whole blood and plasma samples.

Sample preparation

A large number of different methods are available on the market for the extraction of viral DNA. Larger hospital laboratories with high throughput frequently automate the sample preparation step using a robotic workstation while smaller laboratories may use manual extraction kits. When sample numbers are not high enough to justify the investment in an automated system, manual extraction systems based on columns or filters are most often used. Again, the PCR kit must be validated with each individual sample extraction method to ensure accurate and reliable results.

PCR instruments

There are many different real-time PCR instruments available on the market. For PCR reagents to be compatible with different instruments it is very important that the master mix be optimised for the particular instrument being used.

Even differences in geometry of the reaction tube, such as those between capillaries and regular microtubes can affect the reaction to the extent that a given kit may simply not operate on a particular instrument.

Meeting lab requirements

Molecular diagnostic kits based on polymerase chain reaction (PCR) technology are particularly suitable when a sensitive and a quantitative technique is needed for the diagnosis and monitoring of CMV disease. However the overall reliability of the assay depends on the chemistry used in the design of the primers and the number of verification studies that have been carried out.

The overall robustness of the assay is also important and depends not only on the amplification efficiency but also on precision (the day-to-day reproducibility). The physician must be confident in the results in order to define or modify the appropriate treatment for the patient. It is also necessary that the data produced in one laboratory be comparable to those produced in another since optimal patient treatment should not be dependent on geographical location. Raw data also need to be analysed as objectively as possible. This is best done by using appropriate software to further increase the objectivity and reproducibility of the data analysis.

The complete process, from sample preparation through to data analysis

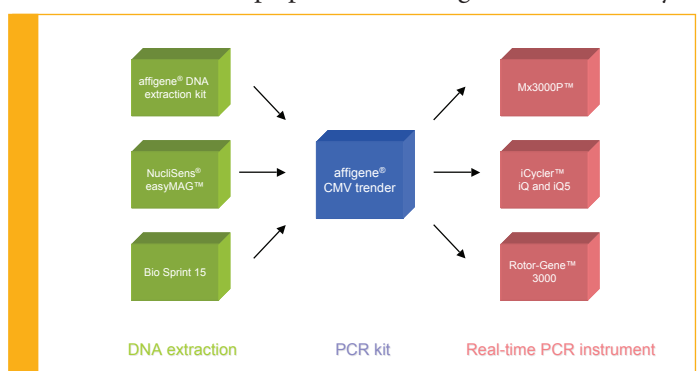


Figure 1. Flexibility concept of affigene® CMV tender system.

needs to be considered when standardising an assay system. Even though the PCR system itself may have been standardised, it is always possible that a combination with a different sample preparation system may negatively affect the results. Examples of such problems include low efficiency of the sample preparation or lack of compatibility of the buffers in the PCR reaction mix with those in the prepared sample being added to the mix. Validated complete processes are therefore vital for reliable overall results.

Standardisation is a tedious process and requires the establishment of many performance characteristics of the kit, such as the limit of detection, precision and specificity. It has been estimated that satisfactory validation of a kit and associated sample preparation method may require the running of up to 600 individual PCR reactions.

Since December 2003 it has been mandatory that molecular diagnostic kits for commercial use be approved via the CE-labelling system. This guarantees that the kit is safe to use and, if used according to the instructions, that the result will be valid for the particular process. The *in vitro* diagnostic directive (IVDD; EC directive 98/79/EC) also requires that the kits actually fulfill the performance claimed for them. However, it is only for the products referred to as high risk (the so-called List A of the Common Technical Specifications — e.g. kits for the detection of HBV, HIV or HCV) that the number of validation studies and samples to be included in the validation process is actually stipulated. The performance of such kits for "high-risk" tests must also be approved externally. Other, non high-risk assays may have performance characteristics that are based on reduced data depending on the particular standards used by each individual manufacturer. However all products must fulfill the essential requirements, which, in addition to other requirements, stipulates that the product must be labelled accurately and that accurate information be included in the user manual.

The affigene® concept for CMV monitoring

The affigene® CMV trender kit, as well as all other affigene® kits, reconciles all regulatory requirements on the one hand and end-customer preferences on the other by being able to be appropriately flexible. As shown in Figure 1, there are three possible DNA sample extraction methods that have been validated for use with this kit. These are (i) a manual

method, using the affigene® DNA extraction kit that is based on silica columns; (ii) the automated NucliSens easyMag workstation from Biomérieux that can handle 24 samples per run and (iii) the Bio Sprint 15 robotic workstation from Qiagen that carries out 15 samples per run. The first two methods have been CE labelled together with the affigene® CMV trender kit. In addition, developments are currently being implemented on a novel instrument that runs 16 samples in parallel and has rapid process times.

The affigene® CMV trender assay can be performed on three different real-time PCR instruments, namely the Mx3000P (Stratagene, La Jolla, USA); the Rotor-Gene 3000 (Corbett Life Sciences, Sydney, Australia) and the iCycler iQ or iQ5 (Bio-Rad, Foster City, USA). The overall process involving the use of any of these instruments with either automated or manual sample preparation systems has been approved for CE-labelling.

The different needs of different sized laboratories are further addressed in the CMV trender kit by the supply of the various reagents in the kit in several vials thus allowing a division of the 48 reactions in the kit into six sets of eight reactions each. In this way, the smaller laboratory that does not run many CMV samples on each occasion will still have fresh reagents for six separate runs. In addition, the supply of the reagents in smaller sets minimises the consequences of any contamination during set-up.

To help the physician to objectively analyse the data produced by the CMV trender test specially-designed affigene® analysis software is available. The software not only handles all controls and standards but also calculates the accurate viral load for each clinical sample. In doing so, the software also takes into account which sample matrix and sample preparation method have been used.

As a result of the considerable effort put into the design and manufacture of the primers and kit components, together with the use of Scorpion technology and the large amount of verification studies performed, the assay system has been shown to provide very high performance characteristics [Table 1].

Sample preparation system	Limit of Detection (c/ml)	Quantitative Range (c/ml)	Precision at LOQ (Standard deviation log ₁₀ (c/ml))
affigene® DNA extraction	88 (61-234)	500 - 10 ⁷	0,17
NucliSens easyMag	57 (36-134)	100 - 10 ⁷	0,17

Table 1. Performance characteristics for affigene® CMV trender on plasma samples prepared on either the NucliSens easyMag instrument or using the affigene DNA extraction. Amplification was performed on the Mx3000P instrument. For limit of detection the 95% confidence interval is shown within brackets.

Conclusion

It is important to monitor patients with suppressed immune systems such as those who have received stem cell transplantation in order to be able to select appropriate treatment with the ultimate goal of reducing morbidity and mortality. For this, a sensitive and quantitative assay is needed. To meet the different demands of different laboratories, the monitoring system should be able to handle different types of sample matrices, sample preparation methods and PCR instruments. Of equal importance is that the process be robust and that the data analysis can be performed objectively so that the final data are reliable. The affigene® CMV trender kit achieves all this.

References

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Foot note: At the time of writing this paper the authors were employed by Sangtec Molecular Diagnostics AB, which was subsequently acquired by Cepheid Inc. Cepheid sells the SmartCMV kit which is CE-labelled for use on the SmartCycler instrument together with the affigene® DNA extraction kit.