

Pitfalls of viral load testing



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Viral load testing is the quantitative measurement of viral nucleic acid in body fluids or tissues. In medical practice, viral load assays are commonly performed for HIV, Hepatitis B and Hepatitis C viruses. These assays are funded under the Medicare Benefits Schedule (MBS) system (Item numbers 69378/81/82, 69482/3 and 69488 respectively; <http://www.health.gov.au/internet/mbsonline/publishing.nsf/Content/Medicare-Benefits-Schedule-MBS-1>).

When is viral load testing performed?

In 2008–2009, 53,592 medicare claims for viral load testing were paid, including approximately 22,500 HIV, 20,000 Hepatitis C and 11,000 Hepatitis B (http://www.medicareaustralia.gov.au/statistics/mbs_item.shtml).

In untreated HIV patients, higher viral loads are associated with an increased rate of CD4 T cell loss and increased risk of progression to AIDS. HIV viral load is used as a tool to monitor patients off therapy, a guide to commencing therapy and a means for monitoring the effectiveness of therapy. In Hepatitis C infections, viral load testing is used as a predictor of response to therapy, with HCV genotype 1 viral loads of less than 600,000 IU/ml being associated with increased rates of successful therapy. Further, patients failing to get a 2 log drop in viral load by week 12 are unlikely to respond to a full course of interferon and ribavirin and are not eligible for Medicare-funded treatment beyond 12 weeks. In Hepatitis B patients, high viral load is associated with increased risk of progression to cirrhosis and hepatoma. HBV viral load testing is funded for the evaluation of patients prior to therapy (the majority of testing) and the monitoring of patients undergoing antiviral therapy.

Viral load testing is important in the management of other medically important viruses. Cytomegalovirus (CMV) viral load measurement is performed either as part of a pre-emptive strategy for the prevention of disease in transplant patients or for monitoring the success of therapy. Polyomavirus BK quantitation in

plasma enables the prediction of BK virus-associated nephropathy in renal transplant patients. EBV quantification in plasma may enable the prediction of post-transplant lymphoproliferative diseases or other B cell lymphomas. However, none of these tests have a specific MBS reimbursement number.

Methods in use in Australia

Like qualitative nucleic acid detection, quantitative nucleic acid detection methods fall into two main groups: target amplification and signal amplification. Target amplification may be achieved by conventional polymerase chain reaction assays, isothermal DNA or RNA amplification assays. However, all quantitative assays must use a standard curve using known starting concentrations of target nucleic acid. This may be from whole organisms, PCR-generated DNA, plasmids or synthetic nucleic acids. Copy numbers may be estimated by measuring the OD of purified nucleic acid, or may be limited to some measure of infectivity (colony-forming units or TCID₅₀). For HIV and Hepatitis B and C, the quantitative assays are standardised against an international standard and results expressed in international units per ml. For example, Hepatitis C RNA copies per ml and converted to IU/ml using conversion factors of 1 IU/ml=2.7 copies/ml for Roche Cobas Taqman and Cobas Amplicor HCV Monitor 2.0 assays and 5.2 copies/ml for the Bayer Versant assay.

A review of the data from each of the Royal College of Pathologists of Australasia (RCPA; <http://www.rcpaqap.com.au/serology>) and National Serology Reference Laboratory, Australia (NRL; http://www.digitalpt.com/catalog/CatalogAU2010_en_Diagnostic_Nucleic_Acid_Testing.pdf) quality assurance programs (QAP) shows that commercial assays using different viral detection techniques and from a variety of manufacturers are in use for the estimation of HIV, HCV, HBV and CMV loads.

The majority of testing is performed using target amplification real-time PCR assays from Roche or Abbotts. Signal amplification assays from Siemens are in use for HIV, but no transcription-mediated amplification assays are used for viral load monitoring. Each of these manufacturers supplies highly automated equipment which is used by the majority of laboratories for the performance of nucleic acid extraction and load testing. In-house assays are uncommon. For example, only one laboratory reported using an in-house quantitative assay (CMV). This reflects the availability of an adequate MBS reimbursement that will cover the cost of the commercial assays plus *de facto* recognition of the difficulty in designing quantitative assays for genetically variable viruses.

Examples of three commercial kits using different technologies for HIV load assay:

- Roche Amplicor HIV-1 Monitor test (*Hoffman-La Roche*), commonly known as the *PCR* test.

- NucliSens HIV-1 QT or NASBA (*bio.Mérieux*).
- Versant/Quantiplex HIV-1 RNA, or bDNA (*Chiron/Bayer*).

What are the problems?

Viral load assays are subject to the same problems as qualitative PCR and any other pathology tests. For summaries of the issues with the development, validation and performance of qualitative and quantitative PCRs, readers are referred to the current National Pathology Accreditation Advisory Council (NPAAC) Laboratory Accreditation Standards and Guidelines for Nucleic acid Detection and Analysis (2006) at: [http://www.health.gov.au/internet/main/publishing.nsf/Content/11E45788D881EE37CA25732B0048D60C/\\$File/NucAcidDetect.pdf](http://www.health.gov.au/internet/main/publishing.nsf/Content/11E45788D881EE37CA25732B0048D60C/$File/NucAcidDetect.pdf) and Draft NPAAC Requirements for Medical Testing of Microorganism Nucleic Acids at: [http://www.health.gov.au/internet/main/publishing.nsf/Content/29E2DC8F21DEF959CA2574E2001DF654/\\$File/Draft%20Micro%20public%20consult-%20June%202919.pdf](http://www.health.gov.au/internet/main/publishing.nsf/Content/29E2DC8F21DEF959CA2574E2001DF654/$File/Draft%20Micro%20public%20consult-%20June%202919.pdf) and a recent review of the validation requirements for in-house nucleic acid test (NAT) testing¹.

Sample preparation

Problems with PCR testing may begin at sample collection and processing. Laboratories must check sample identity and check the samples against request forms and ensure patient identification is not lost during sample manipulation. Sample type, transport and storage, length of time until separation of plasma or serum may impact upon final copy numbers. For HIV, samples spun in the presence of gel but not immediately separated may show small increases in viral load². Fortunately most viruses are relatively stable in serum or plasma at 4°C. During manual aliquotting, samples should be handled separately in a biosafety cabinet.

Nucleic acid extraction

Nucleic acid extraction may vary in efficiency from run to run and should be controlled, for example, by including a human genome target in the nucleic acid testing, or preferably a unique possibly synthetic target in the lysis buffer to monitor extraction efficiency. The same target can be used to monitor for inhibition of the amplification reaction and may provide a correction factor for differing reaction efficiencies in different samples. Purified nucleic acids must be protected from degradation, for example, by RNase inhibitors or storage at -20°C or colder. Low quantities of nucleic acid may need carrier DNA or RNA included to improve extraction efficiency and stability.

Contamination

All amplification reactions may be subject to contamination. Standard molecular laboratory precautions of separating reagent preparation, sample preparation, reaction assembly areas, amplification and detection areas apply. The risk of amplicon contamination is absent in the signal amplification assays. For PCR and other target amplification assays, the use of sealed

reaction wells, real-time fluorescence detection of amplicons, and/or dUTP and Uracil glycosylase in PCR reaction mix also reduces the risk of contamination.

Selection and design of assays

Assay design and quantitation is complicated when the target virus is highly variable. HIV, Hepatitis B and Hepatitis C exist in a number of groups, clades or genotypes and, particularly in the case of RNA viruses, numerous quasispecies within the one host. This creates difficulties for primer and probe design. Highly conserved regions must be targeted and redundancies may need to be included in oligonucleotides or multiple primers and probe sets used to cover the range of virus types. Shorter detection probes utilising duplex stabilising technologies such as LNA bases, 3' minor groove binder (MGB) or 3' BHQplus may enable optimal annealing temperature and stability of the probe-target reaction to be achieved while targeting shorter conserved regions that conventional probe formats could not reliably detect. Where genetic variability is known, the sensitivity of the assay for each of the most common genotypes must be established and monitored. QAP programs must use different genotypes of the viruses.

Conversely, laboratories must be aware of the possibility of lack of amplification or detection when dealing with divergent strains and, where necessary, use alternative assays, which may mean referral to a different laboratory. In order to reliably address this diversity of viral targets, most laboratories have opted to buy a commercial assay rather than develop, validate and maintain an in-house assay.

Assay sensitivity and linearity must be calculated. Assays will generally express a calculated quantitative result within the linear amplification range of the assay. Outside this range, the results are expressed as greater than or less than the linear range. At the lower end, nucleic acid may be detectable but not reliably quantifiable and results expressed as Target Detected (<lower limit of linearity) for example, in the Roche Cobas Ampliprep/Cobas Taqman HCV Test assay

Quality and monitoring

PCR amplification assays are complex in nature and, therefore, inherently variable in their results. Variability in the efficiency of the reaction from run to run should be monitored by the inclusion of quantitative controls. Standardisation of conditions, monitoring of reagents and bulk preparation and pre-release batch testing of reagents will improve assay consistency. These are standards we expect and pay for in commercial assays but should also be included in our in-house assays.

In quantitative assays, a two- to threefold variation in results may be acceptable, particularly with lower target concentration. It is common practice for quantitative results to be expressed in log₁₀ format to simplify the expression of very large numbers but also to reduce concern in the end user of apparent variability of the

results. The RCPA and NRL QAP report results as \log_{10} values including standard deviations (SD) and coefficient of variation (CV). In the RCPA QAP a $\pm 0.25 \log_{10}$ range (less than twofold variation) is one measure used to compare inter-laboratory performance. NRL monitors the day-to-day performance of these assays in different laboratories through its Quality Control (QC) programs using EDCNet (www.nrlqa.net). The QC sample results of one laboratory's HIV and Hepatitis C viral load assays compared to the statistics of other laboratories using the same QC sample and assay are shown as Shewhart graphs in Figure 1. In the NRL Diagnostic NAT QC report of September 2007–2008 SDs of between 0.1 to 0.2 \log_{10} and CVs of between 2% and 8% were seen with a range of HIV, HBV and HCV viral load assays.

Given the complexity of these assays and the target viruses, the intra- and inter-laboratory reproducibility of results is remarkable. It is a testament to the quality of the equipment and

reagents available in the country, the skill of the laboratory staff performing the testing and the efficacy of the quality assurance programs used in Australia.

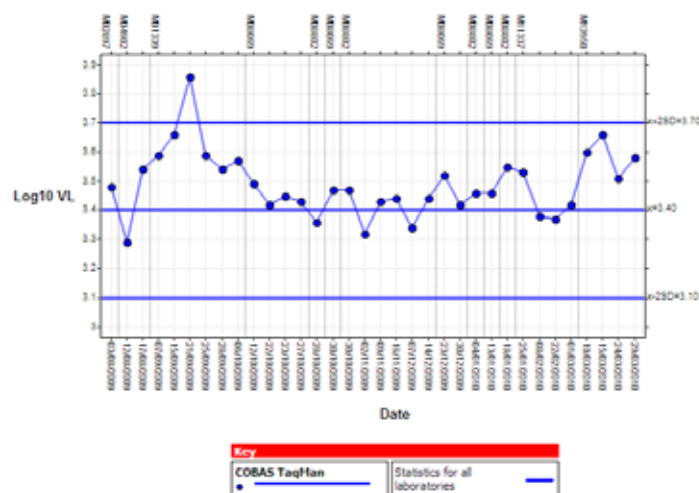
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References

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Results of testing QC sample HIV:QN:118 in the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test for the period of 01/07/2009 to 31/03/2010.



Results of testing QC sample HBV:QN:404 in the Roche COBAS AmpliPrep/COBAS TaqMan HBV Test for the period of 01/04/2010 to 31/07/2010.

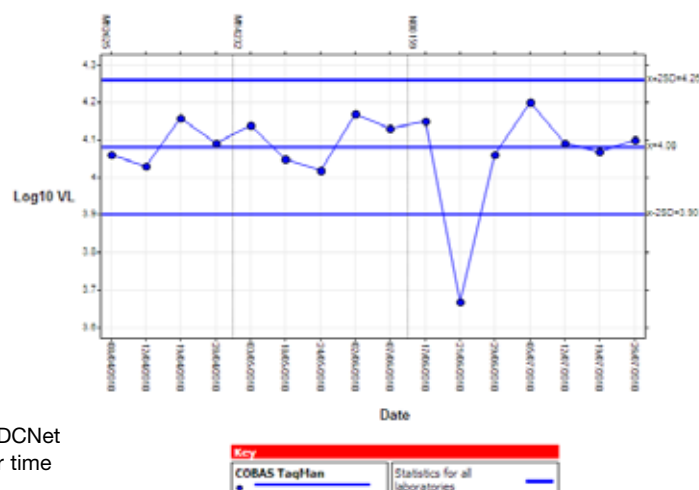


Figure 1: Shewhart plots from EDCNet showing QC sample results over time in HIV and HBV viral load tests.