**Reviewer's report**

**Title:** Development of real-time NASBA assays with molecular beacon detection to quantify mRNA coding for HHV-8 lytic and latent genes

**Authors:**

Abeltje M Polstra (a.m.polstra@amc.uva.nl)
Jaap Goudsmit (j.goudsmit@amc.uva.nl)
Marion Cornelissen (m.i.cornelissen@amc.uva.nl)

**Version:** 1 **Date:** 24 Jun 2002

**Reviewer:** Dr Peter Sillekens

**Level of interest:** A paper whose findings are important to those with closely related research interests

**Advice on publication:** Accept after discretionary revisions

**Report**

The manuscript entitled "Development of real-time NASBA assays with molecular beacon detection to quantify mRNA coding for HHV-8 lytic and latent genes" by Abeltje M. Polstra and colleagues is well written and rather straightforward and consistent. The work described clearly demonstrates the feasibility of quantifying mRNA expression levels in cells using the NASBA amplification technology in combination with molecular beacons for real-time detection. Using these methods, robust assays with a broad range of linearity were obtained for the quantification of several HHV-8 mRNAs. Because only a limited number of patient samples has been studied so far, no firm conclusions can be drawn on the expression patterns for the different mRNAs and their relation to Kaposi's sarcoma. However, the obtained results justify the authors' conclusion that the assays provide valuable tools for further study of HHV-8 replication in patients. Moreover, the methods that have been used and the results that were obtained are described with sufficient level of detail to allow use of this methodology in a broader sense, i.e. for the quantification of mRNAs in general.

**Compulsory revisions:**

1) On page 2 (Results section) the authors state that the limit of quantification (LOD) of 100 molecules was determined for the different assays. On page 9 and onwards it is concluded that the LOQ for each of the assays is 100 molecules. This does not make sense. The authors should clearly state what they find an acceptable accuracy and precision for their assays and subsequently deduce from their data what is the minimum level of RNA that still fulfils these criteria. This minimum level can be regarded as the LOQ for each of their assays. This may well be 100 molecules but in the current version of the manuscript it is unclear how the authors came to this conclusion.

2) A more or less similar reasoning holds true for the limit of detection (LOD). Instead of determining the hit rate at a certain input level (50 copies in the manuscript), it is more common practice to do it the other way around. The authors should define an acceptable hit rate (95% and 50% hit rates are generally accepted levels) and deduce the input level belonging to this hit rate by Probit analyses of their data. Analysis of the data in this way is much more in line with the definition of the LOD of an assay, being the minimum level at which an analyte can be reliably detected.

3) On page 9 (end of "Patient samples" section) the remark that the U1 gene is constitutively expressed,
is irrelevant and confusing. The authors use U1A mRNA as a control. This is the mRNA encoding the so-called A protein, which is one of the proteins of the U1 snRNP particle. In addition to several proteins (one of which is the A protein), this particle also contains U1 snRNA and this is the ground for the confusion. The text should be adapted and reference could be made to literature describing the U1A mRNA to avoid confusion between U1 snRNA and an mRNA encoding one of the proteins that is complexed to this snRNA molecule.

Discretionary revisions:
1) On page 7, the order of magnitude for the primer concentrations in NASBA is uM. On page 8 (top) the primer concentration for the U1A assay is stated as 2 mM. The authors should check whether this should not read uM as well.
2) Specificity is concluded from the absence of any cross-reactivity among the assays( target RNA for a certain assay not being recognized by the primers and/or probes of any of the other assays). It should not be too difficult to demonstrate specificity in a more usual manner, i.e. by analysing a number of HHV-8 negative PBMC specimens.
3) In the absence of an absolute 'golden standard' for the several HHV-8 mRNAs and given the facts that quantitations are based on an external standard curve rather than making use of an internal calibrator in each reaction and that results are 'normalised' based on U1A mRNA levels, an overall conclusion that the assays are semi-quantitative rather than quantitative, would be preferable. In this respect, the authors are also referred to the way in which the results are presented in Table 3. An indication of relative expression levels between the different specimens cq mRNAs is given rather than absolute quantitation results.

Competing interests:

None declared.