Microbiology and Immunology

*Microbiol Immunol* 2018; 62: 673–676
doi: 10.1111/1348-0421.12644

NOTE

# Development of reference material with assigned value for human T-cell leukemia virus type 1 quantitative PCR in Japan

Madoka Kuramitsu[1], Kazu Okuma[1], Makoto Nakashima[2], Tomoo Sato[3], Daisuke Sasaki[4], Hiroo Hasegawa[4], Kazumi Umeki[5], Ryuji Kubota[6], Keiko Sasada[7], Rieko Sobata[8], Chieko Matsumoto[8], Noriaki Kaneko[9], Kenta Tezuka[1], Sahoko Matsuoka[1], Atae Utsunomiya[10], Ki-Ryang Koh[11], Masao Ogata[12], Kenji Ishitsuka[13], Mai Taki[14], Kisato Nosaka[15], Kaoru Uchimaru[2,16], Masako Iwanaga[17], Yasuko Sagara[18], Yoshihisa Yamano[3], Akihiko Okayama[5], Kiyonori Miura[19], Masahiro Satake[8], Shigeru Saito[20], Toshiki Watanabe[2,21] and Isao Hamaguchi[1]

[1]Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, [2]Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo, [3]Department of Rare Diseases Research, Institute of Medical Science, St. Marianna University School of Medicine, Kawasaki, [4]Department of Laboratory Medicine, Nagasaki University Hospital, Nagasaki, [5]Department of Rheumatology, Infectious Diseases and Laboratory Medicine, University of Miyazaki, Miyazaki, [6]Division of Molecular Pathology, Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, [7]Department of Laboratory Medicine, Kumamoto University Hospital, Kumamoto, [8]Central Blood Institute, Blood Service Headquarters, Japanese Red Cross Society, [9]Department of Infection and Immunology, SRL Inc., Tokyo, [10]Department of Hematology, Imamura General Hospital, Kagoshima, [11]Department of Hematology, Osaka General Hospital of West Japan Railway Company, Osaka, [12]Department of Hematology, Oita University Hospital, Oita, [13]Division of Hematology and Immunology, Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, [14]Rakuwakai Kyoto Medical Examination Center, Kyoto, [15]Department of Hematology, Kumamoto University of Medicine, Kumamoto, [16]Department of Hematology and Oncology, Research Hospital, Institute of Medical Science, The University of Tokyo, Tokyo, [17]Department of Frontier Life Science, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, [18]Japanese Red Cross Kyushu Block Blood Center, Fukuoka, [19]Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, [20]Department of Obstetrics and Gynecology, University of Toyama, Toyama and [21]Department of Advanced Medical Innovation, Graduate School of Medicine, St. Marianna University, Kawasaki, Japan

## ABSTRACT

**Quantitative PCR (qPCR) of human T-cell leukemia virus type 1 (HTLV-1) provirus is used for HTLV-1 testing and for assessment of risk of HTLV-1-related diseases. In this study, a reference material was developed for standardizing HTLV-1 qPCR. Freeze-dried TL-Om1 cells diluted with Jurkat cells were prepared and an assigned value for proviral load (PVL) of 2.71 copies/100 cells was determined by digital PCR. Nine Japanese laboratories using their own methods evaluated the PVLs of this reference material as 1.08–3.49 copies/100 cells. The maximum difference between laboratories was 3.2-fold. Correcting measured PVLs by using a formula incorporating the assigned value of this reference material should minimize such discrepancies.**

**Key words** human T-cell leukemia virus type 1, proviral load, quantitative PCR, standard.

Human T-cell leukemia virus type 1 was the first human retrovirus to be discovered (1, 2). It has a worldwide distribution with some endemic areas, including Japan (3, 4). HTLV-1 is transmitted through breastfeeding, sexual contact and blood contact, such as transfusion or injection of HTLV-1-positive blood. Some carriers of HTLV-1 develop ATL, HTLV-1-associated myelopathy/tropical spastic paraparesis or

**Correspondence**
Isao Hamaguchi, Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashimurayama, Tokyo, Japan. Tel.: +81 42 561 0771; fax: +81 42 565 3315; email: 130hama@niid.go.jp

Received 20 April 2018; revised 8 August 2018; accepted 14 August 2018.

**List of Abbreviations:** ATL, adult T cell leukemia; HTLV-1, human T-cell leukemia virus type 1; PVL, proviral load'; qPCR, quantitative PCR.

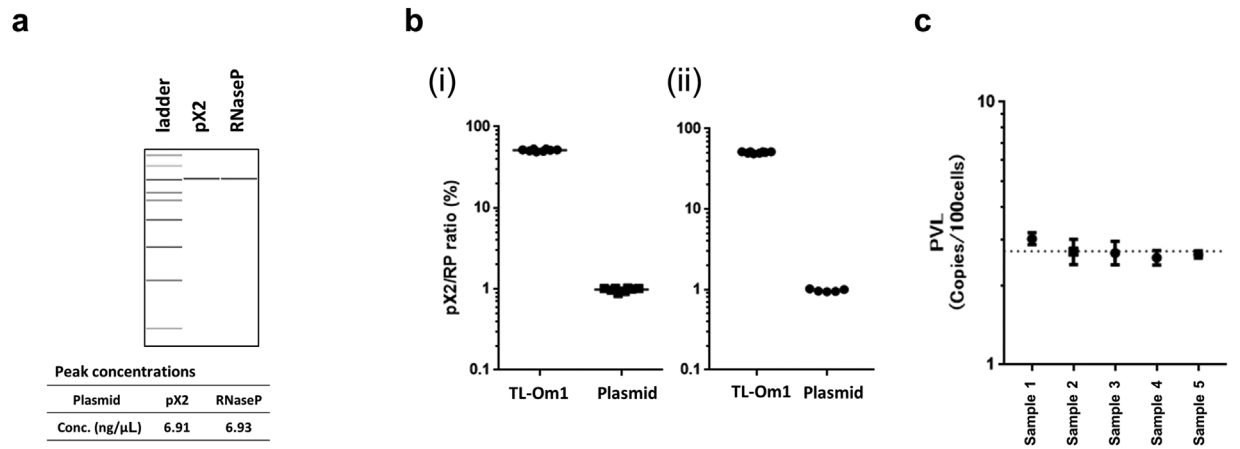


**Fig. 1. Digital PCR analysis of reference for HTLV-1 qPCR.** (a) Plasmid DNA containing HTLV-1 target sequence (pX2: middle lane) or internal control gene (RNaseP: right lane) were electrophoresed and the bands quantitated using Bioanalyzer. (b) Ratios of HTLV-1 (pX2) to RNaseP in TL-Om1 genomic DNA and 1% w/w plasmid mixture (pX2:RNaseP = 1:100) were analyzed using two types of digital PCR machines, (i) QX100 and (ii) QuantStudio 3D ($n = 8$). (c) PVLs of the reference were measured by droplet digital PCR. Genomic DNA extracted from five different tubes was subjected to droplet digital PCR ($n = 9$). PVLs (copies/100 cells) were calculated as follows: (pX2 copy number × 2)/(RNaseP copy number) × 100. The dotted line (PVL at 2.71 copies/100 cells) is the geometric mean of the five samples.

HTLV-1 uveitis/HTLV-1-associated uveitis after long incubation periods (5). qPCR, which detects the integrated provirus in the peripheral blood of carriers, is a conventional method for measuring the HTLV-1 PVL. Importantly, a high PVL is reportedly one of the risk factors for development of ATL and HTLV-1-associated myelopathy. Accurate PVL quantitation is therefore essential; however, current results differ by around five-fold between laboratories (6).

International standards for nucleic acid amplification tests for blood-borne pathogens such as hepatitis B and C viruses and HIV have been developed by the World Health Organization and made available worldwide by the National Institute for Biological Standards and Control (7–9). However, international standards for nucleic acid amplification tests for HTLV-1 have not yet been developed. Because Japan is one of the endemic areas of HTLV-1, reference material, even of a prototypic format, is urgently needed to enable accurate performance of HTLV-1 qPCR.

We have previously reported that TL-Om1, an HTLV-1 infected cell line derived from ATL, is suitable for creating a reference material for HTLV-1 qPCR and that it would be possible to standardize HTLV-1 qPCR using TL-Om1 (10). In this study, we prepared a freeze-dried cell reference material composed of TL-Om1 cells diluted with Jurkat cells to serve as a temporary reference for HTLV-1 qPCR and evaluated its quality with the collaboration of Japanese laboratories.

Because we had previously found that Jurkat cells are much more viable than frozen peripheral blood mononuclear cells, we selected Jurkat cells for preparation of the reference material (11). TL-Om1 cells were diluted with Jurkat cells at a concentration of approximately 1% and aliquots freeze-dried (see Supporting Materials and Methods). A value was assigned to the PVL of the reference material as determined by digital PCR. First, to confirm the accuracy of the digital PCR result, we measured a test sample made by mixing two plasmids, the first containing a HTLV-1 qPCR target sequence (pX region of HTLV-1 [pX2]) and the other an internal control gene target sequence (RNase P, [RP]). As shown in Figure 1a, the size and amount of the plasmids were confirmed to be the same. The plasmids were mixed at a concentration of 1.0% w/w (pX2/RP) (Fig. 1a). The concentration of the test sample was then measured by droplet digital PCR (QX-100) and chip-based digital PCR (QuantStudio 3D) as 0.98% and 0.99%, respectively (Fig. 1b and Table 1), confirming that the digital PCR results were extremely close to the absolute value. Under these conditions, the PVL of the reference material was determined to be 2.71 copies/100 cells (95% CI, 2.49–3.41) (Fig. 1c).

**Table 1.** Ratio of pX2 (HTLV-1) to RNaseP (internal control gene) (%) as analyzed by digital PCR

| | QX100 | | QuantStudio 3D | |
|---|---|---|---|---|
| | Geometric mean | 95% CI | Geometric mean | 95% CI |
| TL-Om1 | 51.3 | 50.1–52.6 | 51.0 | 49.9–52.0 |
| 1:100 plasmid | 0.98 | 0.94–1.03 | 0.97 | 0.93–1.02 |

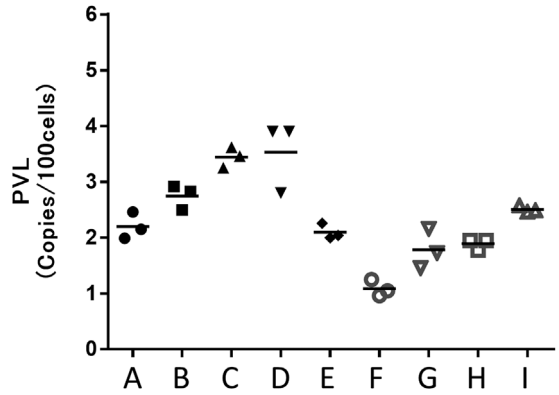


**Fig. 2.** Proviral loads of the reference measured collaboratively. qPCR was performed independently three times on different days. Horizontal axis: the letters A to I denote the laboratories that participated in this study. Bars, geometric means.

Next, the PVL of the reference material was measured by nine Japanese laboratories using their in-house methods. The results ranged from 1.08 to 3.49 copies/100 cells (Fig. 2 and Table 2), the maximum difference being 3.23-fold between laboratories and the geometric mean of the PVLs being 2.23 copies/100 cells. The ratio to assigned value ranged from 0.40 to 1.29, revealing a wide discrepancy among laboratories, as had been observed previously (11). However, correcting these PVLs by using a formula incorporating the assigned value of the reference material should minimize these discrepancies. To confirm this, PVLs of clinical samples were measured by different laboratories. The geometric coefficient of variation (%) between laboratories was less in most clinical samples after making this correction (Fig. 3 and Table 3). These results indicate that inter-laboratory variations can be minimized by correction with the formula incorporating the assigned value of the reference material. Finally, all participants agreed to

**Table 2.** PVL as measured by qPCR at nine Japanese laboratories

| Laboratory | Geometric mean | 95% CI of geometric mean | Ratio to assigned value |
|---|---|---|---|
| A | 2.19 | 1.68–2.86 | 0.81 |
| B | 2.74 | 2.23–3.37 | 1.01 |
| C | 3.44 | 3.01–3.94 | 1.27 |
| D | 3.49 | 2.17–5.62 | 1.29 |
| E | 2.10 | 1.78–2.47 | 0.77 |
| F | 1.08 | 0.77–1.51 | 0.40 |
| G | 1.76 | 1.08–2.87 | 0.65 |
| H | 1.89 | 1.65–2.16 | 0.70 |
| I | 2.51 | 2.36–2.66 | 0.93 |

the formula and the assigned value (2.71 copies/100 cells) of the HTLV-1 qPCR reference material.

Unlike standards for hepatitis B and C viruses and HIV, the target for HTLV-1 qPCR is not viral particles within plasma, but provirus integrated into the genomes of host cells. Cell-based material rather than serum or plasma is therefore more suitable for use as a HTLV-1 qPCR reference. We used the Jurkat cell line for diluting TL-Om1 cells because, as we have reported previously, the karyotypes of internal control genes are close to 2N, which is useful for preventing discrepancies that may be generated by the use of different internal control genes in different laboratories (10).

In this study, we succeeded in preparing a reference material for HTLV-1 qPCR. Although we made relatively small amounts of this reference material using laboratory equipment, we believe we will be able to provide a continuous supply by renewing the lot periodically. We also plan to evaluate the long-term stability of the lyophilized reference material by regularly measuring its PVL using digital PCR. Inter-laboratory differences in qPCR should be dramatically decreased by correcting results by using the assigned value of the reference. The reference material could also be used to

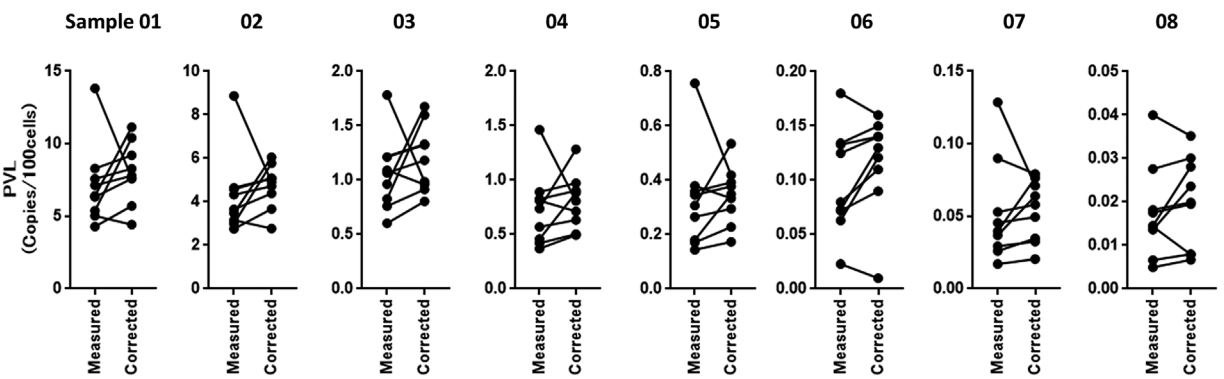


**Fig. 3.** Proviral loads of clinical samples before and after correction with the assigned value of the reference. Clinical samples with various PVLs were measured by nine laboratories. Dots show PVLs of measured values (left) and corrected values (right). Corrections were made by dividing by the ratio between the measured and assigned values of the reference.

**Table 3.** Geometric coefficient of variations of clinical samples among laboratories

| Sample ID | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 |
|---|---|---|---|---|---|---|---|---|
| Measured values | | | | | | | | |
| PVL (geometric mean) | 6.7 | 4.0 | 1.0 | 0.67 | 0.29 | 0.086 | 0.043 | 0.015 |
| GCV (%) | 35% | 36% | 32% | 46% | 54% | 67% | 68% | 71% |
| Corrected values | | | | | | | | |
| PVL (geometric mean) | 7.7 | 4.6 | 1.2 | 0.77 | 0.33 | 0.097 | 0.050 | 0.017 |
| GCV (%) | 29% | 24% | 26% | 32% | 35% | 93% | 49% | 71% |

GCV, geometric coefficient of variations; PVL: copies/100 cells.

minimize discrepancies arising from the use of different methods, such as qPCR and digital PCR. Additionally, it will be useful for preparation of further nucleic acid amplification test standards for HTLV-1.

## ACKNOWLEDGMENTS

We thank Harutaka Katano, DDS, PhD, and Hideki Hasegawa, MD, PhD, (Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan) for help with the droplet digital PCR technique. We also thank Sarah Williams, PhD, from Edanz Group (www.edanzediting.com) for editing a draft of this manuscript. This work was supported by a Health Labor Sciences Research Grant [H23-sinkou-ippan-016] and the Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development, AMED [H26-sinkoujitsuyouka-ippan-013].

## DISCLOSURE

The authors declare they have no conflicts of interest.

## REFERENCES

1. Hinuma Y., Nagata K., Hanaoka M., Nakai M., Matsumoto T., Kinoshita K.I., Shirakawa S., Miyoshi I. (1981) Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc Natl Acad Sci USA* **78**: 6476–80.
2. Poiesz B.J., Ruscetti F.W., Gazdar A.F., Bunn P.A., Minna J.D., Gallo R.C. (1980) Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* **77**: 7415–9.
3. Gessain A., Cassar O. (2012) Epidemiological aspects and world distribution of HTLV-1 infection. *Front Microbiol* **3**: 388.
4. Satake M., Yamaguchi K., Tadokoro K. (2012) Current prevalence of HTLV-1 in Japan as determined by screening of blood donors. *J Med Virol* **84**: 327–35.
5. Watanabe T. (2011) Current status of HTLV-1 infection. *Int J Hematol* **94**: 430–4.
6. Kamihira S., Yamano Y., Iwanaga M., Sasaki D., Satake M., Okayama A., Umeki K., Kubota R., Izumo S., Yamaguchi K., Watanabe T. (2010) Intra- and inter-laboratory variability in human T-cell leukemia virus type-1 proviral load quantification using real-time polymerase chain reaction assays: a multi-center study. *Cancer Sci* **101**: 2361–7.
7. Baylis S.A., Heath A.B.; Collaborative Study Group. (2011) World Health Organization collaborative study to calibrate the 3rd International Standard for Hepatitis C virus RNA nucleic acid amplification technology (NAT)-based assays. *Vox Sang* **100**: 409–17.
8. Davis C., Berry N., Heath A., Holmes H. (2008) An international collaborative study to establish a replacement World Health Organization International Standard for human immunodeficiency virus 1 RNA nucleic acid assays. *Vox Sang* **95**: 218–25.
9. Fryer J.F., Heath A.B., Wilkinson D.E., Minor P.D., Collaborative Study G. (2017) A collaborative study to establish the 3rd WHO International Standard for hepatitis B virus for nucleic acid amplification techniques. *Biologicals* **46**: 57–63.
10. Kuramitsu M., Okuma K., Yamagishi M., Yamochi T., Firouzi S., Momose H., Mizukami T., Takizawa K., Araki K., Sugamura K., Yamaguchi K., Watanabe T., Hamaguchi I. (2015) Identification of TL-Om1, an adult T-cell leukemia (ATL) cell line, as reference material for quantitative PCR for human T-lymphotropic virus 1. *J. Clin Microbiol* **53**: 587–96.
11. Kuramitsu M., Okuma K., Yamochi T., Sato T., Sasaki D., Hasegawa H., Umeki K., Kubota R., Sobata R., Matsumoto C., Kaneko N., Naruse I., Yamagishi M., Nakashima M., Momose H., Araki K., Mizukami T., Mizusawa S., Okada Y., Ochiai M., Utsunomiya A., Koh K.R., Ogata M., Nosaka K., Uchimaru K., Iwanaga M., Sagara Y., Yamano Y., Satake M., Okayama A., Mochizuki M., Izumo S., Saito S., Itabashi K., Kamihira S., Yamaguchi K., Watanabe T., Hamaguchi I. (2015) Standardization of quantitative PCR for human T-cell leukemia virus Type 1 in Japan: A collaborative study. *J Clin Microbiol* **53**: 3485–91.

### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

**Supporting materials and methods.** Materials and methods used in this report.
**Supporting references.** References cited in supporting materials and methods.