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An International Collaborative Study to establish a WHO Internal Standard for *Toxoplasma gondii* DNA Nucleic acid amplification technology assays

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by
4 October 2014 and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr David Wood** at email: *woodd@who.int*.

Summary
Seventeen laboratories from 14 countries participated in an international collaborative study to establish a WHO International Standard for *Toxoplasma gondii* DNA nucleic acid amplification technology (NAT) assays. In all, 20 separate data sets were collected from these laboratories. Five samples, AA which was lyophilised and BB, CC, DD and EE which were liquid preparations, were analysed using several different NAT assays. The mean *T. gondii* DNA content of each sample was determined from the study. The mean $\log_{10}$ "equivalents"/ml were 6.0 for sample AA, 5.91 for sample BB, 2.88 for sample CC, 3.01 for sample DD and 6.48 for sample EE. Predictions of the stability of the freeze-dried preparation AA indicate that it is extremely stable and suitable for long term use. On the basis of the collaborative study data and the results of the stability studies, the freeze-dried material, AA is proposed the first International Standard for *T. gondii* DNA NAT assays. The code number of AA is 10/242 and the proposed potency is $1x10^6$ International units per ml based upon this study. Each vial contains the equivalent of 0.5 ml of material, and the content of each vial would be $5x10^5$ IU/ml.

Introduction
Toxoplasmosis is caused by the protozoan *Toxoplasma gondii.* Infection usually results in a self-limiting lymphadenopathy but ocular disease is observed in both healthy and immunocompromised individuals (1) and causes more severe life-threatening disease in the immunocompromised (2) and through congenital infection (3). The parasite is transmitted by the ingestion of raw meat or water containing *T. gondii* tissue cysts or oocysts respectively. Transmission can also occur vertically during pregnancy and there have been documented cases of transmission through transplanted organs (4). Felines are the definitive host for *T. gondii* (5) and oocysts excreted from the feline intestinal tract can survive for up to 18 months in the environment (6). The parasite replicates asexually in nucleated cells in most other vertebrate hosts (5).

The prevalence of the human population infected with *Toxoplasma* varies widely from country to country (10 to 80% or 10 to 98%) (7,8). Low seroprevalences are observed in North America, South East Asia and Northern Europe whereas high seroprevalences are observed in Latin America and tropical Africa.

Detection of *Toxoplasma gondii* is carried out using serological methods, both IgM and IgG, by histological examination of infected tissues, by PCR of infected body fluids or by culture of the parasite (9). All methods have advantages and disadvantages. In this study we are focusing on PCR detection of *T. gondii*. Real-time PCR assays are sensitive and effective for the detection of *T. gondii* however inter assay variations are common. These differences may be a result of target gene chosen, intrinsic variability in assay sensitivity or as a consequence of calibration using different reference reagents that are poorly standardised. Due to this variability the proposed International Standard will be expressed in International Units. These arbitrary units can be used to compare assays or laboratories by the response given by the standard.

This preparation will be intended for the comparison of the sensitivities of NAT based methods for the detection of *T. gondii*. It is not subject to requirements for the manufacture and control of biological substances. However, the need for this material has been recognised by the ECBS in 2009.

The first WHO International Standard for NAT assays was established in 1997 for hepatitis C virus (HCV) RNA (10). This standard was crucial in enabling the introduction of screening of blood donations and plasma pools for HCV RNA by NAT assays. Further WHO International Standards for NAT assays have been established for hepatitis B virus (HBV) DNA, HIV-1 RNA, parvovirus B19 DNA, hepatitis A virus (HAV) RNA (11, 12, 13, 14). These standards have all been widely used in the implementation of NAT testing to ensure the safety of blood and plasma derived medicinal product with respect to contamination with blood borne viruses. The first International Standard for *Plasmodium falciparum* DNA was established in 2006 (15). This was the first parasitology NAT International Standard produced.

The objectives of this present study were to assess the suitability of a candidate preparation as a WHO International Standard for *T. gondii* DNA for use in NAT assays and to determine *T. gondii* DNA content of candidate standard.

In this study, 17 laboratories from 14 countries returned results from assays of four separate preparations. These included parasitology laboratories from tropical medicine institutes, universities, hospitals and other laboratories. All assays were in-house assays developed by the respective laboratories or from published methods. Throughout this report, a code number was allocated at random and did not necessarily represent the order of the participants described in Appendix 1 to the participants. Data sheets and a method form were provided so that all relevant information could be recorded. The collaborative study began in April 2013 and concluded in March 2014.

Bulk Material and Processing:

Preparation and evaluation of materials

Five candidate materials were included in this study. Sample AA was a freeze-dried preparation of *T. gondii* tachyzoites harvested from mice in antibiotic saline and then diluted in a buffer containing 30 mg/ml Trehalose and 10mM Tris buffer. The final concentration of *T. gondii* was approximately 1×10^6 parasites/ml, based on microscopy analysis by trained operators. Sample AA was stored at -70ºC until filling and freeze-drying. Sample BB was a frozen liquid bulk of the material used to produce sample AA. Sample CC was a 1:1000 dilution of sample AA in cerebro spinal fluid containing approximately 1×10^3 parasites/ml. Sample DD was a 1:1000 dilution of sample AA in amniotic fluid containing approximately 1×10^3 parasites/ml. Sample EE was a frozen liquid bulk of *T. gondii* tachyzoites of the RH strain harvested from tissue culture cells (Hela cell line) in serum free medium. The final concentration of *T. gondii* was approximately 2.4×10^6 parasites/ml.

Lyophilisation of sample AA

Sample AA was filled and freeze-dried at Standards Processing Division, NIBSC, UK. The material was processed as follows: on the day of the fill, the bulk sample was thawed with constant agitation until the sample had just thawed out. Thereafter, the bulk material was kept at 5.2 to 6.6°C. A total of 3218 vials were filled with 0.5 ml of material. The co-efficient of variation of the fill volume was 0.211, determined by measuring every twenty-eighth vial ampoule. Measurements were made for a total of 114 ampoules.

The ampoules were washed without detergent prior to sterilisation by oven baking at 180°C for 3 hours. Rubber seals were immersed in 95% ethanol, 5% methanol for a minimum of one hour followed by autoclaving. The seals were then placed on top of the filled ampoules before being loaded into the freeze-drier.

The shelves of the freeze-drier were pre-cooled to -40°C prior to the loading of the vials. The temperature was maintained at -40°C for at least 3 hours in the absence of any vacuum. After this initial period, a maximum vacuum was applied, whilst maintaining shelf temperature at -40°C, for a period of 90 hours. The condenser temperature was at or below -70°C. After this period the temperature was raised from -40°C to +20°C over 20 hours while maintaining maximum vacuum. Maximum vacuum was maintained for a further 2-6 hours at +20°C before the freeze-drying chamber was back filled with N_2 and the vials sealed within the freeze-drier. A moisture trap was positioned between the N_2 and freeze-drier to ensure dryness. N_2 gas with less than 5ppm O_2 was used. The vials were removed from the freeze-drier, crimp sealed with aluminium over seals and stored at -20°C. Freeze-drying was completed on the 25th of January 2011. Lyophilised vials of sample AA are stored at -20ºC with constant temperature monitoring, at the National Institute for Biological Standards and Controls (NIBSC). The CV of the dry-weight content was 2.74%. The residual moisture content for 10/242 has been determined as 0.2667%. The oxygen content of the product has been determined as 0.45%. Residual moisture and oxygen content levels are determined since they can impact upon the stability of freeze-dried preparations. All manufacturing records are held by NIBSC to be available on request by the ECBS.

Stability Studies

Stability studies were performed at NIBSC for sample AA. Vials of AA were stored at -20°C, +4°C, +20°C and +37°C. Vials from each temperature were removed after different intervals and initially stored at -70°C before analysis. In order to provide a "baseline" titre against which to compare the stability of the samples incubated at different temperatures, vials of AA, stored continuously at -70°C, were analysed in parallel.

For analysis of the samples for the stability study, 1000 µl volumes of the reconstituted samples for AA were extracted using the COBAS Ampliprep (Roche Applied Science). Samples were extracted using the COBAS Ampliprep TNA Isolation Kit (Roche Applied Science) according to the manufacturer's instructions. The final matrix was eluted in 100 µl of elution buffer. Real-time PCRs were performed on the LightCycler 480 instrument (Roche Applied Science, Mannheim, Germany). An in-house assay was performed using primers and probe selected to detect the *T. gondii* AF 487550 gene. The forward primer sequence was as follows: 5_-AGA GAC ACC GGA ATG CGA TCT-3'. The reverse primer sequence was as follows: 5_-CCC TCT TCT CCA CTC TTC AAT TCT-3. The sequence of the fluorogenic hydrolysis Taqman probe was as follows: 6FAM-ACG CTT TCC TCG TGG TGA TGG CG-TAMRA. Amplification reactions were performed using the LightCycler FastStart DNA Master Hybprobe kit (Roche Applied Science, Mannheim, Germany) and 5 µl of the eluted DNA was used in a 20 µl reaction volume. The concentration of each primer in the reaction was 0.6 µM and the probe was used at a final concentration of 0.2 µM. The amplification conditions were as follows: 95ºC for 10 min, then 45 cycles of the following sequential steps: 95 ºC for 15s, 60 ºC for 1 min. Fluorescence data was collected during the combined annealing/extension step and detected at 530 nm. A standard curve was generated using serial ten-fold dilutions of a sample with a concentration of 1 x 10^6 parasites/ml. This was

determined by enumeration of parasites by light microscopy, by a proficient operator, with independent confirmation.

The potency estimates for each temperature at the different times are below. Potency is based on measuring a mean of triplicate estimates for each temperature against the baseline temperature of -70°C.

Time (months)	Temp (°C)	Potency
12	-20	0.6807
12	4	0.7389
12	20	1.4551
12	37	1.4158
18	-20	0.8286
18	4	0.9396
18	20	0.8508
18	37	0.9030
30	-20	0.8092
30	4	0.7768
30	20	0.7413
30	37	0.7768

Because of the variability in these estimates and no obvious degradation between temp/time the Arrhenius model was unable to fit to the data and looking at the data it is concluded that there is no observable degradation for the samples.

Design of the Study

Participants were sent four vials of each material, AA-EE, shipped on dry-ice. The participants were requested to store samples at -70°C on receipt. Participants were requested to re-dissolve the lyophilised preparation (AA) in 0.5ml of nuclease –free deionised water immediately before use. The vials were to be shaken gently occasionally over a period of 20 minutes to dissolve the contents. The liquid preparations (BB, CC, DD, EE) were to be thawed quickly before use.

Participants were requested to perform four independent assays for the detection of *T. gondii* DNA on the samples AA-EE on different days, preferably one week apart if possible. A fresh vial of each sample was to be used for each independent assay. For each assay dilutions of all 5 preparations were to be tested at the same time so that the *T. gondii* DNA content of each preparation could be determined. Participants were requested to prepare dilutions in the sample diluent normally used in their assay system.

For the first assay of the 5 preparations, participants were requested to assay ten-fold dilutions of the preparations in order to determine the *T. gondii* DNA end-point of each preparation. For the remaining 3 assays it was suggested that participants should half-log dilutions (i.e. 1:3.3) either side of the end point. Data sheets were provided record all results and list data required for each assay, such as extraction methods, amplification methods, etc. A separate data sheet was used for each assay. A detailed protocol for the study is shown in Appendix 3.

Statistical Methods

The qualitative end-point assays were analyzed using the Poisson model, as previously described for collaborative studies for other NAT standards (10, 11, 12, 13, 14). All estimates were expressed as $\log_{10}$ PCR detectable units/ml. Overall means were calculated as arithmetic means of the $\log_{10}$ estimates.

The quantitative assays were expressed as Laboratory Mean Estimated Parasites/ml ($\log_{10}$) where parasite concentrations were given or as Estimated NAT-detectable units/ml ($\log_{10}$) calculated from qualitative end-point dilution assays.

Potencies relative to sample AA were calculated as the difference in estimated $\log_{10}$ detectable units/ml.

Data Received

Data Received and Exclusions:

Data were received from 17 laboratories. They are referred to by a code number, allocated at random, and not reflecting the order of listing in the appendix. Six laboratories returned data from assay methods described as quantitative. The other laboratories returned data from assays described as qualitative.

Quantitative Assays:

Of the six laboratories with quantitative assays, three (3, 10 and 14) returned estimates of parasites/ml, based on a local reference. The other three laboratories (7, 12 and 16) returned Ct values for the dilution series tested.

The dilutions quoted by laboratory 7 for sample BB differ from those for sample AA, and are inconsistent with the stated concentration of parasites. Analysis has been performed based on the quoted dilution factor. The Ct values appear inconsistent at Ct values higher than around 32/33. It was not possible to use the Ct values for samples CC and DD to obtain estimates of potency relative to AA.

For the other laboratories only Ct values from the top 1 – 3 concentrations of samples CC and DD could be used for analysis, as the higher Ct values became inconsistent at lower concentrations.

Qualitative Assays:

For laboratory 1, assay 4 had higher responses than the other 3 assays with no end-point obtained. All assays were included in subsequent analysis however. Sample EE was positive at all dilutions tested in all assays, and no estimate of NAT-detectable units/ml could be obtained.

For laboratory 2, the qualitative positive or negative classification was done with a conventional PCR, while Ct values were provided from an RT PCR.

Laboratory 5 used limited dilution ranges for assays 2-4 (2 repeats at +/- 0.5 log, rather than single repeats at +/-0.5 and +/- 1.0 log). This may lead to some additional variability in the estimation of

NAT-detectable units/ml. The fluorescence values returned did not appear suitable for further parallel-line analysis.

For laboratory 6 sample EE was positive at all dilutions tested in all assays, and no estimate of NAT-detectable units/ml could be obtained.

Laboratory 8 did not provide a classification of positive or negative, and the Ct values were beyond the linear portion of the dose-response curve in most cases. No estimates of NAT-detectable units, or potencies relative to sample AA, could be obtained.

Laboratory 13 used different assay methods and/or targets for each of their four assays. The results could not therefore be pooled to estimate NAT-detectable units from an end point dilution series. The end-points quoted by the laboratory are included in the tables. Parallel line analysis of the Ct values from assays 3 and 4 were used to obtain potency estimates relative to sample AA. These are referred to in the results section by the codes 13C and 13D. However, for sample AA, the Ct values quoted were identical for both assays, so it is possible that one set is incorrect.

Laboratory 15 returned results from assays using three different PCR target genes. The results are referred to as 15A (B1-A), 15B (B1-B) and 15C (Rep529). A positive/negative classification was not given explicitly, but Ct values quoted as >40 were taken as negative, and anything with a stated Ct was taken as positive.

For laboratory 17, the dilutions used, or results obtained, for sample EE across assays appear inconsistent. No estimate of NAT-detectable units/ml could be obtained for sample EE.

Results

Parasite Counts:

Only three laboratories (3, 10 and 14) returned estimated parasite counts. The mean of the $\log_{10}$ count was calculated across assays for each dilution, for each laboratory and sample, and corrected for dilution factor. For laboratory 10, the corrected estimates were non-linear across the range of dilutions used, with differences of up to 1 log. For example, for sample A, the mean counts/ml were 5.45 $\log_{10}$ based on the undiluted sample, rising to 6.53 $\log_{10}$ for the 10^{-4} dilution. The mean for the 10^{-2} dilution was 6.14 $\log_{10}$. This pattern was observed for all samples.

For laboratories 3 and 14, the estimated counts (corrected for dilution factor) appear linear across the dilutions for samples AA to DD. For sample EE laboratory 3 had slightly lower estimates for samples tested neat (around 0.3 log) compared to results tested at higher dilutions. Laboratory 14 had the reverse pattern with estimated counts decreasing at higher dilutions by up to 0.9 $\log_{10}$.

Overall means for each sample and laboratory were calculated using the data from all dilutions in each case, as there was no specific justification for excluding any of the data. The results are shown in table 1

The results for samples CC and DD are most variable between laboratories, with up to 0.8 log difference between labs. Overall means for the candidate standard AA and the liquid bulk BB are very close at 6.12 and 6.13 $\log_{10}$ parasites/ml respectively. Lab 14 has higher estimates than other labs for samples AA and BB, but the results are more variable for the other samples.

NAT-detectable units from qualitative end-point assays:

The qualitative end-point assays were analysed using the Poisson model, as previously described for collaborative studies for other NAT standards (10). All estimates were expressed as $\log_{10}$ NAT detectable units/ml. Overall means were calculated as arithmetic means of the $\log_{10}$ estimates.

The estimated $\log_{10}$ NAT-detectable units/ml from the qualitative end-point assays are presented in table 2, and are also presented in histogram form in figures 1a-e. Each box represents the estimate from one laboratory, and is labelled with the laboratory code number. The overall means, and between laboratory standard deviations (SD) are also shown in table 2.

The mean NAT-detectable units/ml for all samples are generally around one log higher than the estimated parasites/ml from the three laboratories shown in table 1. Sample EE is a little higher than samples AA and BB, while samples CC and DD are around 3.0 log lower. This pattern is consistent with the estimates of parasites/ml from table 1.

The overall mean $\log_{10}$ NAT detectable units/ml for sample AA is higher than for sample BB (7.47 $\log_{10}$ for sample AA compared to 7.25 $\log_{10}$ for sample BB). This is probably due to assay variability, as the number of NAT-detectable units is not expected to increase on freeze-drying. Laboratories 2, 11 and 15B in particular have higher estimates for AA than for BB. The estimation of NAT-detectable units from end-point dilution assays is intrinsically variable.

The histograms for samples CC and DD (figs 1c and 1d) suggest that the results for sample CC are higher than for sample DD. The overall means are 4.15 and 4.42 $\log_{10}$ NAT-detectable units/ml respectively. This difference is marginally significant using a paired t-test ($p=0.049$) but not when using a non-parametric alternative (Wilcoxon signed rank test; $p=0.067$).

The variability between laboratories is highest for samples AA, BB and EE, compared to samples CC and DD (standard deviations in table 2). This may be related to the choice of dilutions used for the higher concentration samples.

Quantitative Assays:

Six laboratories returned data from assays described as quantitative. Three provided estimates of parasites/ml as described above and shown in table 1, along with relevant CT values. Three further laboratories (7, 12 and 16) only provided Ct values for dilution series of samples AA – EE, and so no absolute quantitation of the samples (in either parasites/ml or NAT-detectable units/ml) is possible. For all six laboratories, a parallel line analysis of Ct against log concentration was performed, to express the potencies of samples BB-EE relative to sample AA, taking sample AA as 10^6 units/ml.

Potencies relative to Sample AA:

The potencies of samples BB-EE relative to sample AA were calculated assuming an assigned value of 10^6 units/ml for sample AA. For the qualitative end-point assays, the relative potency was defined by the difference in $\log_{10}$ NAT-detectable units/ml between the sample and sample AA, corrected for the nominal value of 6.0 $\log_{10}$ units/ml for sample AA. For the quantitative assays, the results

from the parallel line analysis of Ct values were used. The results are presented in table 3. They are also shown in histogram form in figure 2. The results from the quantitative assays are shaded in grey.

For quantitative assays, the agreement between labs is reasonable, particularly for sample BB. The exception is lab 7, which has very variable assay results, and no dose-response for either samples CC or DD. For sample BB, they had estimates of 6.25, 3.59 and 5.94 from assays 1, 2 and 4 respectively (no estimate from assay 3 due to non-linearity of Ct values for BB). There is no indication why the estimates from assay 2 are so low, but the Ct values are much higher than for AA at the equivalent dilutions.

Analysis of Ct values from qualitative end-point assays:

Some laboratories returned Ct values along with the positive/negative classification for their qualitative assays. Where the samples were tested at sufficient dilutions to give reasonable dose-response curves (Ct against log concentration), these were also analysed as parallel line assays. For most laboratories this was only possible for the first assay, which generally included a wider range of dilutions. The Ct values for subsequent assays, with dilutions targeted around the positive/negative end-point, showed insufficient linear dose-response to allow further analysis. For two laboratories (2 and 4) the parallel line analysis was possible for all 4 assays.

The resulting potencies of samples BB-EE relative to sample AA (taking sample AA as 10^6 units/ml) are shown in table 4. 13C and 13D refer to the assays 3 and 4 from lab 13, which used a different method for each assay. However, the Ct values reported for sample AA were identical for both assays.

The relative potencies calculated from the Ct values are broadly similar to those calculated from the estimated NAT-detectable units/ml, considering that they are mainly based on single assays, and that the estimation of NAT detectable units from end-point assays is intrinsically variable.

Laboratory 13 has much lower estimated potencies for samples CC and DD than for other laboratories. As each assay was done with a different method, it was not possible to determine an estimate of NAT-detectable units/ml using the Poisson model, and no comparison is possible. Laboratory 13 did return a summary table of their detection limits for each sample and assay method, which is shown in table 5. The values are "Number of *Toxoplasma* cells in tested sample". There does appear to be a big difference in sensitivity between samples CC and DD, compared to AA or BB. For example, for assays 3 & 4 the detection limits for AA and BB are 10^{-2} cells, compared to 5 or 50 for CC and DD.

Overall mean estimated potency relative to Sample AA:

The overall mean and between laboratory variability (as measured by the SD) for laboratory estimates of the potencies of samples BB-EE relative to sample AA (taking sample AA as 10^6 units/ml) are shown in table 6. This table is split into three sections, showing the potencies calculated by the different methods described above, namely – Parallel Line analysis of Ct values from Quantitative assays; Based on NAT-detectable units calculated from end-point qualitative assays; Based on limited analysis of Ct values from qualitative assays, where possible. The results from laboratory 13 were not included in this third category. As noted above (and table 4), their results for samples CC and DD were completely different from other laboratories (by 10^3), and there

were doubts over the reported Ct values for sample AA (which were quoted as identical for two different assays).

The between laboratory variability in estimates of relative potency is generally smallest for the quantitative assays. This is as expected. The estimates of NAT-detectable units/ml from end-point assays is intrinsically variable, leading to relatively high variability between laboratories for the relative potency estimates calculated from the NAT-detectable units. Where it has been possible to perform a limited (parallel line) analysis of the Ct values from the qualitative assays, the agreement between laboratories is improved. However, this comparison is based on data from different subsets of laboratories and assays.

The estimated relative potencies, in $\log_{10}$ units/ml, are in reasonably good agreement for the different methods of calculation, given the variability anticipated for estimates based on NAT-detectable units. It is important to note that within assay variability is a key factor in any discrepancies between potencies for BB compared to AA with some labs reporting AA to be more concentrated than BB. The mean estimate for sample BB from the quantitative assays is influenced by the low value obtained from laboratory 7 (above and table 4). If the result from laboratory 7 were excluded, the overall mean and between laboratory SD would be 6.03 and 0.04 $\log_{10}$ for sample BB. This represents very good agreement between laboratories. The comparison of AA and BB represents a 'best case' situation as the materials are the same apart from AA undergoing lyophilisation. The close agreement between AA and BB (overall means within 0.03 $\log_{10}$ excluding lab 7) also suggest that the lyophilisation had little impact on the estimated parasites/ml or NAT-detectable units/ml.

The overall estimated mean potencies relative to AA for samples CC and DD are also in close agreement, with similar between laboratory variability. These samples were frozen liquid samples containing the same number of *T. gondii* diluted in either CSF or Amniotic fluid. These results suggest that the different matrices have not made a difference to the effectiveness of expressing the results relative to the candidate IS, sample AA.

For sample EE, the inter-assay variability of the qualitative end-point assays is improved by expressing results relative to sample AA, but for samples CC and DD this is not the case as the variability has increased. This is a result of correcting the results for CC and DD by the results for sample AA, which were variable between laboratories (fig 1a). The best agreement is obtained from the quantitative assays, using a parallel-line analysis of the Ct values. (See Table 6)

All laboratories in this study used one or both of 2 DNA sequences as their target sequence for the NAT assays. These targets are the B1 gene and a 529-bp repeat sequence seen in the *T. gondii* genome. The B1 gene contains 35 copies/parasite while the novel 529-bp repeat contains between 200-300 copies/parasite. This could lead to variability between results. However comparing the data for laboratories in Table 1 there is close agreement in concentrations with laboratories10 and 14 using assays targeting the 529-bp repeat whereas laboratory 3 used an assay targeting the B1 gene. All 3 laboratories used their own in-house standards to construct their standard curves. This demonstrates the usefulness of an International Standard in ensuring coherence between laboratories.

Conclusions

Based on the collaborative study data, the freeze-dried material, AA is proposed as the first International Standard for *T. gondii* DNA NAT assays. The code number of AA is 10/242 and the proposed potency is 10^6 International Units per ml based upon the results of the study. Each vial contains the equivalent of 0.5 ml of material, and the content of each vial would be 5×10^5 IU. The Instructions for Use for 10/242 are shown in Appendix 4. Predictions of the stability of the freeze-dried preparation AA indicate that it is extremely stable and suitable for long term use.

Responses from Participants

All participants were in favour of this report and stressed the value of this potential standard.

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Appendix 1: List of Participants

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Appendix 2: Tables and Figures

Table 1
Laboratory Mean Estimated Parasites/ml (log_{10})

Lab	Sample				
	AA	**BB**	**CC**	**DD**	**EE**
03	5.99	5.90	2.90	2.97	6.42
10	6.04	6.05	3.73	3.71	6.19
14	6.36	6.47	3.50	3.62	6.47
Mean	**6.12**	**6.13**	**3.41**	**3.46**	**6.37**

Table 2
Estimated NAT-detectable units/ml (log_{10}) calculated from qualitative end-point dilution assays.

Lab	Sample				
	AA	**BB**	**CC**	**DD**	**EE**
01	6.29	6.70	3.81	3.71	-
02	9.46	8.56	4.91	4.68	-
04	7.13	7.56	4.06	4.06	8.31
05	6.47	6.97	4.37	4.39	7.06
06	7.96	8.08	3.86	4.64	-
09	8.07	7.32	4.21	4.64	9.07
11	7.92	6.65	4.60	4.57	8.03
15A	6.99	6.67	3.79	4.10	6.67
15B	7.44	6.68	4.06	4.39	7.33
15C	7.06	7.16	3.86	4.93	7.14
17	7.36	7.37	4.16	4.50	-
Mean	**7.47**	**7.25**	**4.15**	**4.42**	**7.66**
SD	0.88	0.62	0.36	0.34	0.84

Table 3

Potencies relative to sample AA, assuming arbitrary assignment of 6.0 $\log_{10}$ units/ml for AA

Assay	Lab	Sample			
		BB	CC	DD	EE
Quantitative	03	6.03	2.93	3.00	6.52
	07	5.26	-	-	6.94
	10	5.97	3.05	3.03	6.21
	12	6.04	3.21	3.18	5.79
	14	6.06	3.28	3.40	6.11
	16	6.05	2.62	2.77	6.58
Qualitative	01	6.41	3.52	3.42	
	02	5.09	1.45	1.22	
	04	6.43	2.93	2.93	7.18
	05	6.51	3.91	3.92	6.59
	06	6.12	1.90	2.68	
	09	5.25	2.14	2.57	7.00
	11	4.73	2.68	2.65	6.11
	15A	5.68	2.80	3.11	5.68
	15B	5.25	2.62	2.95	5.89
	15C	6.09	2.80	3.87	6.08
	17	6.00	2.79	3.13	

Table 4
Potencies relative to sample AA, assuming arbitrary assignment of 6.0 $\log_{10}$ units/ml for AA
Based on analysis of Ct vales from Qualitative Assays

Lab	Sample			
	BB	CC	DD	EE
01	5.91	-	-	6.33
02	6.11	2.24	2.00	7.51
04	5.98	2.94	3.07	6.56
09	5.78	2.74	2.59	6.53
11	5.95	3.44	3.03	6.54
13C	6.66	0.74	0.43	-
13D	6.65	0.96	0.80	-
15A	6.29	3.32	3.45	6.93
15B	6.18	3.03	3.25	6.79
15C	6.09	2.95	3.34	6.66
17	6.08	2.67	3.15	-

13C and 13D refer to the assays 3 and 4 from lab 13, which used a different method for each assay. However, the Ct values reported for sample AA were identical for both assays.

Table 5
Detection limits summarised by laboratory 13 for their four assay methods

Sample	Assay 1 single round PCR, B1 gene	Assay 2 single round PCR, REP gene	Assay 3 Real-time PCR, B1 gene	Assay 4 Real-time PCR B1 gene
AA	10^{o} (1)	10^{o} (1)	10^{-2}	10^{-2}
BB	10^{-2}	10^{-2}	10^{-2}	10^{-2}
CC	50	5	5	50
DD	50	0,5	0,5	50
EE	dilution x 5	dilution x 6	dilution x 6	dilution x 5

The values are “Number of Toxoplasma cells in tested sample”

Table 6

Overall mean potencies relative to sample AA (log_{10} units/ml), assuming arbitrary assignment of 6.0 log_{10} units/ml for AA

	Potencies Relative to AA Calculated from										
		Quantitative Assays Ct values				Qualitative End-Point Assays NAT detectable units				Qualitative Assays* Ct values		
Sample		n	Mean	SD		n	Mean	SD		n	Mean	SD
BB		6	5.90	0.32		11	5.78	0.61		9	6.04	0.15
CC		5	3.02	0.26		11	2.69	0.69		8	2.92	0.38
DD		5	3.08	0.23		11	2.95	0.73		8	2.99	0.47
EE		6	6.36	0.41		7	6.36	0.57		8	6.73	0.36

n – Number of Labs.
Mean – Overall mean (log10) across labs.
SD – Standard deviation of log10 estimates between labs.

* Excluding results from 13C and 13D

Figure 1

NAT-detectable units/ml (log_{10}) estimated from qualitative end-point dilution assays

Figure 1a – Sample AA

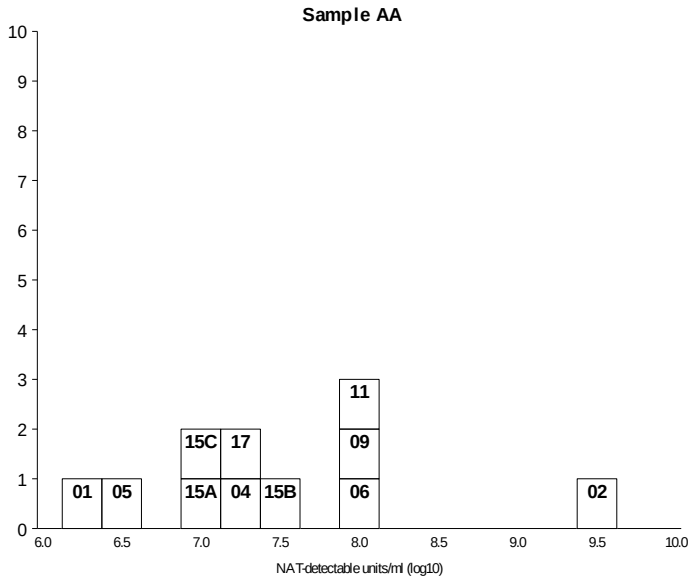


Figure 1b – Sample BB

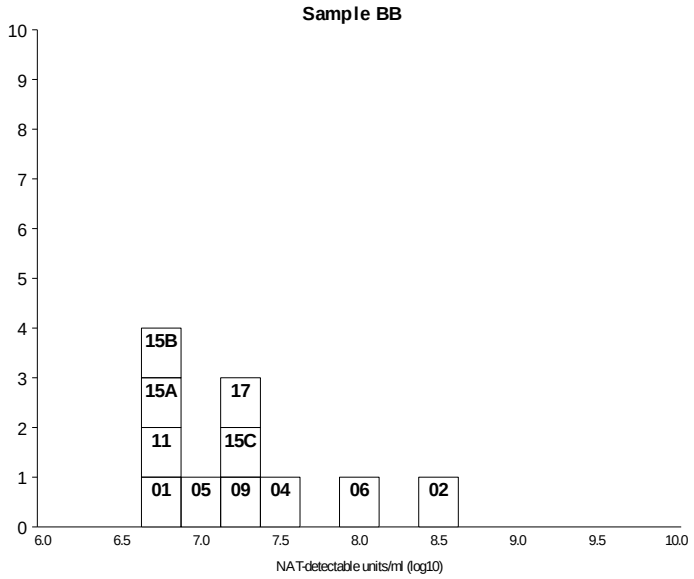


Figure 1c – Sample CC

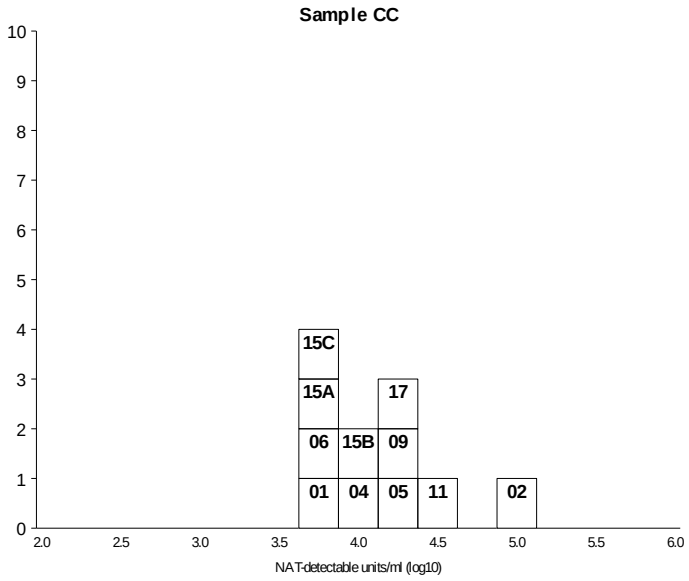


Figure 1d – Sample DD

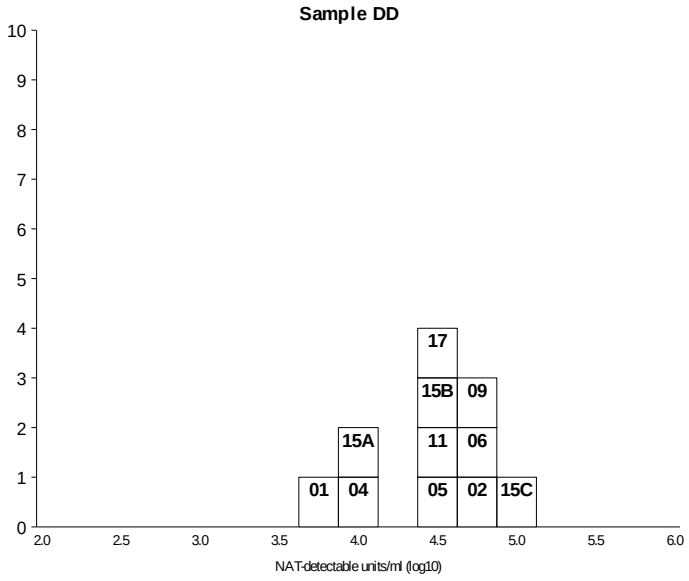


Figure 1e – Sample EE

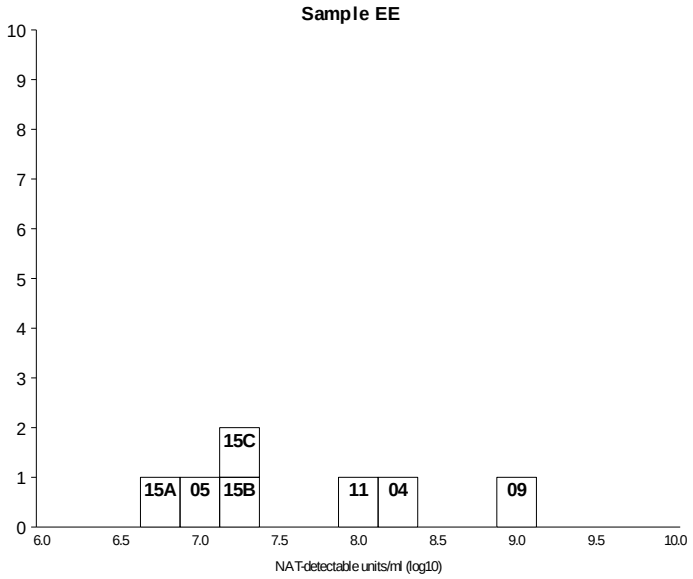


Figure 2

Potencies relative to Sample AA (log10 Units/ml) based on assigned 6.0 log10 units/ml for AA

Results based on quantitative assays shaded in grey, and results based on qualitative end-point dilution assays (NAT-detectable units) clear (white).

Figure 2a – Sample BB

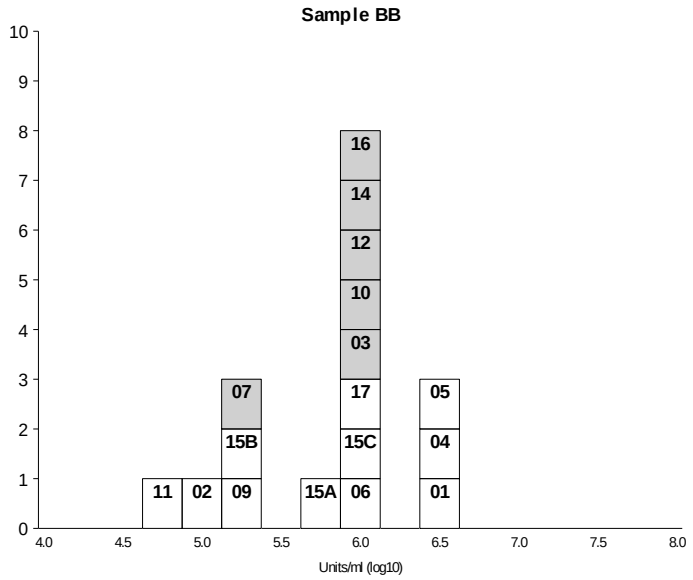


Figure 2b – Sample CC

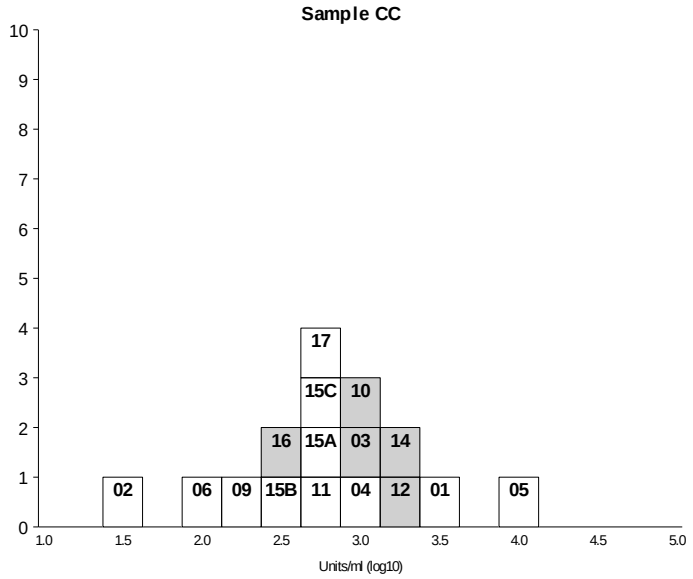


Figure 2c – Sample DD

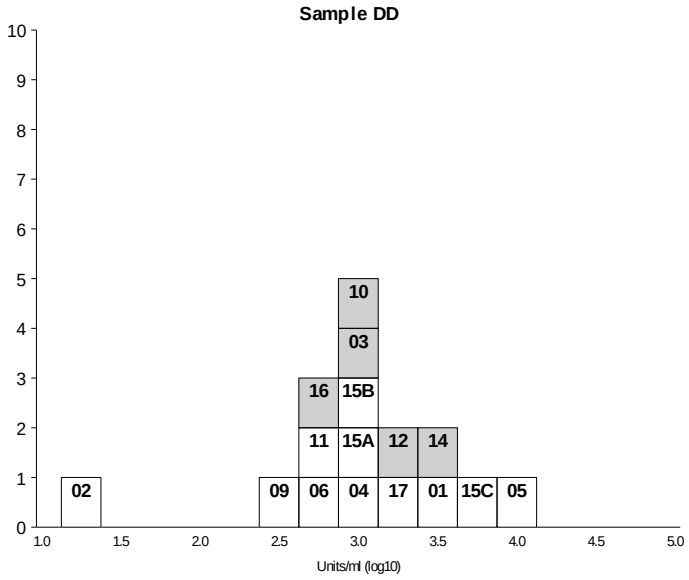
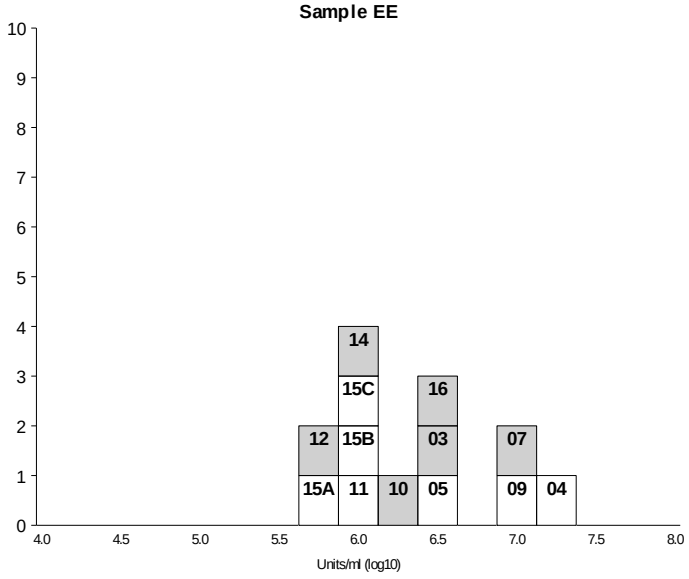


Figure 2d – Sample EE



Appendix 3: Collaborative study for the characterisation of an international standard for *Toxoplasma gondii* nucleic acid amplification assays.

COLLABORATIVE STUDY FOR THE CHARACTERISATION OF AN INTERNATIONAL STANDARD FOR *Toxoplasma gondii* NUCLEIC ACID AMPLIFICATION ASSAYS.

Objectives:

1. To assess the suitability of a candidate preparation as an International Standard for *Toxoplasma gondii* DNA for use in nucleic acid amplification techniques (NAT).
2. To determine the unitage of the candidate standard.

Materials:
Sample AA is a 0.5ml lyophilised sample of *Toxoplasma gondii* containing approximately 1x 10^6 parasites per ml.
Sample BB is a frozen liquid sample of *Toxoplasma gondii* containing approximately 1x 10^6 parasites per ml.
Sample CC is a frozen liquid sample of *Toxoplasma gondii* in Cerebro Spinal Fluid containing approximately 1x 10^3 parasites per ml.
Sample DD is a frozen liquid sample of *Toxoplasma gondii* in Amniotic Fluid containing approximately 1x 10^3 parasites per ml.
Sample EE is a frozen liquid sample of *Toxoplasma gondii parasites derived from tissue culture.*

CAUTION

THESE PREPARTIONS ARE NOT FOR ADMINISTRATION TO HUMANS.

The preparations contain *Toxoplasma gondii* parasites. **These preparations are not considered infectious material as they have been inactivated and screened for Hepatitis B & C, HIV1/2 and Syphilis. However, they should be used and discarded according to your own laboratory safety procedures. Care should be exercised in opening vials to avoid cuts.**

Design of the Study:

Participants will be sent 4 vials of each material. All samples should be stored at -70°C on receipt. The lyophilised preparations should be re-dissolved in 0.5ml of deionised nuclease-free water immediately before use. The water should be delivered to the vial with a 1ml sterile, disposable syringe fitted with an appropriate sterile, disposable needle (08.mm x 25mm is suitable). The vial should be shaken gently over a period of 20 min to dissolve the contents. The liquid preparations may be thawed quickly in a water bath at 37°C or at room temperature.

Participants are requested to perform four independent assays for *Toxoplasma gondii* of the 5 samples on different days. A fresh vial of each material should be used for each independent assay. For each assay, dilutions of all 5 candidate materials should be tested at the same time so that the

Toxoplasma gondii DNA content of each preparation can be determined. **Participants should prepare dilutions in the sample diluent normally used in their assay system. All dilutions should undergo DNA extraction prior to PCR amplification. Dilutions should not be made post-extraction.**

For the first assay of the 5 candidate materials, participants using **Qualitative** assays should assay ten-fold dilutions of the preparations in order to determine the *Tox*oplasma *gondii* DNA end-point of each preparation.
In the remaining **Qualitative** 3 assays, half-log dilutions (i.e. 1:3.3 dilutions) on either side of the end-point should be assayed.
For **Quantitative** assays it is necessary to assay down to 10^{-4} and record the relevant concentrations and Ct values. This should be carried out for the 3 subsequent assays.

Results:

Data sheets are provided so that all relevant information can be recorded. A separate data sheet should be completed for each assay.

Result forms for the *Toxoplasma gondii* DNA Collaborative Study

Assay 1:

Sample	Dilution	Positive/Negative or Concentration	Ct value	Comments
AA				
BB				
CC				

DD				
EE				

Result forms for the *Toxoplasma gondii* DNA Collaborative Study

Assay 2:

Sample	Dilution	Positive/Negative Or Concentration	Ct value	Comments
AA				
BB				
CC				

DD				
EE				

Result forms for the *Toxoplasma gondii* DNA Collaborative Study

Assay 3:

Sample	Dilution	Positive/Negative Or Concentration	Ct value	Comments
AA				
BB				
CC				

DD				
EE				

Result forms for the *Toxoplasma gondii* DNA Collaborative Study

Assay 4:

Sample	Dilution	Positive/Negative Or Concentration	Ct value	Comments
AA				
BB				
CC				

DD				
EE				

Method Form *Toxoplasma gondii* Collaborative Study

Investigator: Institute:

Type of Institute (Control Authority, Manufacturer, Diagnostic Laboratory, Kit Manufacturer, Other laboratory (please specify)):

Qualitative assays
In-house developed PCR
Commercial assay: Version: Batch:

Quantitative assays
In-house quantitative assay
Commercial quantitative assay: Version: Batch:

Protocol for *Toxoplasma gondii* DNA isolation from samples

Indicate reference if the method has been published:
Journal: Volume: Year: Pages:

Volume used for extraction:
Volume of final elute:
Indicate to which category the extraction method belongs and specify the reagents
Chaotropic agent (e.g. guanidium isothiocyanate, urea, LiCl):
Chaotropic agent with phenol/chloroform extraction:
Chaotropic agent with absorption to silica or ion exchange resin:
Proteinase K digestion and phenol/chloroform extraction:
Commercially available method: Manufacturer:
Name of the method: Batch:

Other in house developed methods, please describe briefly:

Sample diluent used:

Amplification protocol

Volume of amplification reaction:
Volume of template in amplification reaction:
Indicate reference if the method has been published:
Journal: Volume: Year Pages:

The amplification procedure can be categorised as:
Single round PCR
Nested PCR
Other procedure, describe briefly:

Genomic region amplified and copy number if known:

Describe primer sequences applied, please give the exact sequences in A, T, G and C
First round PCR primer: Forward:
Reverse:

Nested PCR: Forward (outer):
Reverse (outer):
Forward (inner):
Reverse (inner):

Characteristics of the PCR-protocol:
DNA polymerase used: Manufacturer:

Thermal cycler manufacturer:
No. of cycles 1st round: No. of cycles 2nd round:

Protocol for detection of amplification products

Amplification products have been detected by:
Gel electrophoresis followed by ethidium bromide staining and UV-visualisation
Gel electrophoresis followed by nylon or nitro-cellulose filters and hybridisation with a radio-active labelled probe
Gel electrophoresis followed by blotting on nylon or nitro-cellulose filters and hybridisation with a enzyme-labelled or chemiluminescent probe
Using fluorescent, chemiluminescent or enzyme labelled primers and/or probes
Oligomer-hybridisation (liquid hybridisation) followed by separation of hybridised probe-amplification product and non-hybridised probe on gels

Dot-blot analysis on filters by radioactive probe detection
Dot-blot analysis on filters by enzyme labelled or chemiluminescent probe detection
Other method, describe briefly or give literature reference:

In the case of hybridisation describe the probe and associated labels used

For oligomers, please give the exact sequences in A, T, C, and G.
Probe sequences:

Quality assurance

Is an internal control used to account for inhibition or loss of nucleic acid? Yes No
Please describe details of the internal control if used:

Please give exact sequence in A, T, G, and C.

Are weak positive samples included in each run for monitoring sensitivity?
Are negative samples included in each run for monitoring specificity?

No of controls per test run: positive control:
negative control:

Are isolation, amplification and detection performed in separate areas? Yes No
Is a method for prevention of PCR carry-over used? Yes No
If yes describe the method:

Appendix 4: Instructions for Use for 1st WHO International Standard for *Toxoplasma gondii* DNA Nucleic Acid Amplification Techniques

WHO International Standard 1st WHO International Standard for *Toxoplasma gondii* DNA NAT Assays NIBSC code: 10/242 Instructions for use (Version 1.00, Dated 06/06/2014

1. INTENDED USE

The WHO International Standard for *Toxoplasma gondii* DNA nucleic acid amplification technology (NAT) assays consists of a freeze-dried whole blood preparation collected from a patient by exchange transfusion. The standard has been lyophilized in 0.5 ml aliquots and stored at -20ºC. The material was calibrated in an international collaborative study involving 17 laboratories.

2. CAUTION

This preparation is not for administration to humans.

The material is not of human or bovine origin. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

This material has been assigned a unitage of 5 x 105 International Units (IU) per vial
Uncertainty: the assigned unitage does not carry an uncertainty associated with its calibration. The uncertainty may therefore be considered to be the variance of the vial content and was determined to be +/- 2.74%

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial contains 0.5 ml of lyophilized prepartion containing *Toxoplasma gondii* tachyzoitesof RH strain in a buffer containing 30 mg/ml Trehalose and 10mM Tris.

5. STORAGE

The *Toxoplasma gondii* DNA International Standard should be stored at – 20ºC or below until use.

Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an ‘easy-open’ coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
The material is supplied lyophilized and before use should be reconstituted in 0.5 ml of sterile nuclease-free water. The reconstituted material has a final concentration of 1 x 10^6 IU/ml. If all the reconstituted material is not used immediately, laboratories may aliquot the remaining material into suitable volumes which should be stored at -70ºC.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

10. ACKNOWLEDGEMENTS

11. FURTHER INFORMATION
Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_qu estions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION
In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET Physical and Chemical properties

Physical appearance: Freeze-dried		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2

Other (specify):

Toxicological properties

Effects of inhalation: Not established, avoid inhalation

Effects of ingestion: Not established, avoid ingestion

Suggested First Aid

Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water.
Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS
In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of

Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY
Country of origin for customs purposes*: United Kingdom
* Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 6 g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable**.**
Attached: No

17. CERTIFICATE OF ANALYSIS
NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_bi olefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

Appendix 5: Comments made by participants of the collaborative study.