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Collaborative Study to Establish the 1st World Health Organization International Standard for Mycoplasma DNA for Nucleic Acid Amplification Technique (NAT)-Based Assays

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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 04 October 2013 and should be submitted electronically to the Responsible Officer: Dr Ana Padilla at email: padillaa@who.int, with a copy to Dr David Wood at email: woodd@who.int.

Contents

Summary

This report describes the World Health Organization (WHO) project to develop an International Standard (IS) for mycoplasma DNA for the use with nucleic acid amplification technology (NAT) based test kits.

The international collaborative study consisted of two parts:

(a) an initial study to investigate the feasibility of standardization or harmonization in the field of mycoplasma NAT-based assays and, if successful, for selection of the candidate preparation (feasibility study), and

(b) a comparability study for value assignment to the candidate preparation.

The feasibility study revealed that a mycoplasma species may be representative for other, even distantly related mycoplasma species facilitating the harmonization of NAT assays designed for generic mycoplasma detection. As a result of these studies, the WHO IS candidate chosen is a culture-based preparation of *Mycoplasma fermentans* which has been freeze-dried for long-term stability.
The freeze-dried candidate standard was characterized in parallel with the original liquid bulk material to evaluate potential effects on DNA detectability introduced by the lyophilization process; no detrimental effects were observed.
Stability studies have been initiated, and interim data obtained under accelerated conditions already indicate long-term stability of the analyte.

It is proposed to establish the 1st World Health Organization International Standard for mycoplasma DNA for nucleic acid amplification technique (NAT)-based assays with a unitage of 2×10^5 IU/ml.

Introduction

The detection of different species of mycoplasmas by nucleic acid amplification technology (NAT)-based assays (NAT assays) is playing an increasing role both in the testing of materials used for the manufacture of biopharmaceutical products and for the differential diagnosis of bacterial infections in humans or animals. Throughout this report we use the term "mycoplasmas" or "mycoplasma species" as the trivial name for the bacteria in class Mollicutes, as it is in common use in many publications.
Regulations in different parts of the world (e.g. Europe, USA, and Japan) increasingly accept NAT assays for testing biological materials for mycoplasma contamination, either with NAT replacing culture tests or with NAT being performed as a supplement to culture assays.[1] These NAT assays are usually designed for generic detection by equivalent amplification of distantly related mycoplasma species.

It has been shown that NAT assays of different design may result in quite different qualitative or quantitative results[2], and these differences may be due to different assay features and/or to lack of standardization of assay. The World Health Organization (WHO) standardization program for biologicals aims to harmonize diagnostic methods by the use of a common reference preparation, e.g. a WHO International Standard (IS), which may be assigned an arbitrary unitage, the International Unit (IU). If possible, WHO ISs are produced as freeze-dried preparations to facilitate distribution worldwide without requiring a cold chain. The approach of establishing freeze-dried preparations containing the target analyte of biological origin has been proven successful in many fields, e.g. for the harmonization of diagnostic and screening NAT assays for blood borne viruses like hepatitis viruses (including HBV, HCV, HEV) or HIV.

The Paul-Ehrlich-Institut (PEI, Langen, Germany) is a "WHO Collaborating Centre for Quality Assurance of Blood Products and in vitro Diagnostic Devices" and initiated the project to develop a WHO IS for mycoplasma NAT assays, endorsed by the WHO Expert Committee on Biological Standardization (ECBS) in October 2010.
The collaborative study summarized in this report consisted of two parts: 1) a feasibility study to investigate potential harmonization of assays and to select a candidate material; 2) a follow-up comparability study to characterize the candidate WHO IS and to assign an International Unitage.

Both parts of the collaborative study were presented and discussed in different scientific forums. The idea behind the project was first discussed at the Parenteral Drug Association (PDA) Mycoplasma Task Force meeting in 2009 in Berlin and in several subsequent Task Force meetings since. The project was also reviewed at the 3rd Clinical Diagnostics meeting of the Scientific Working Group on the Standardization of Genome

Amplification Techniques (SoGAT) in London (UK) in January 2011 and at the joint 24th Blood Virology and 4th Clinical Diagnostics meeting of SoGAT in Ljubiljana (Slovenia) in May 2013.

Feasibility Study. The feasibility study was designed to investigate if a preparation containing a mycoplasma strain would improve harmonization of NAT assays designed for generic mycoplasma DNA detection. The study was also intended to select a suitable mycoplasma species for further development as the candidate WHO IS.
In 2012, the feasibility study was performed with worldwide representation of different mycoplasma NAT assays. Volunteer laboratories representing regulatory authorities, manufacturers of biological medicinal products, IVD manufacturers and contract research organizations received coded panels of lyophilized specimens representing four mycoplasma species: *Acholeplasma laidlawii*, *Mycoplasma (M.) fermentans*, *M. orale* and *M. pneumoniae*. These four species represent distant branches of the phylogenetic tree of the bacteria class Mollicutes (Figure 1).
For the study, quantitative NAT assays were defined as assays reporting analyte concentrations in a unitage (e.g. "copies/ml", "genome copies/ml", "genomes/ml"), after respective calibration of the assays.
Semi-quantitative NAT assays were defined as reporting signal intensities which correlate with the concentration of the analyte, e.g. threshold cycle (C_T) values of real-time PCR.
Semi-quantitative or quantitative assays were differentiated from qualitative NAT assays reporting test results as either "positive" or "negative".
Results were evaluated by the PEI after all participating laboratories had completed the study. The feasibility study report, proposing a *M. fermentans* preparation as the most suitable WHO IS candidate material, was distributed to participants. There were no objections to the proposal, and where comments were received from the participants these were incorporated into the final report, as appropriate.

Comparability study. After completion and evaluation of the feasibility study, the selected WHO IS candidate material (Panel Member 8; *M. fermentans*) was prepared under identical conditions as those used for the production of the feasibility study panel members. The candidate material was characterized in terms of residual moisture and for potential effects introduced by the lyophilization process. A stability study under accelerated conditions was started. The lyophilized WHO IS candidate preparation was characterized by preselected NAT assays of different design, in parallel with Panel Member 8 from the feasibility study. Based on results of the comparability study and on potency estimates for Panel Member 8, the candidate material was assigned a value in IU/ml close to the mean "copies/ml" values determined for the quantitative assays and the mean "NAT detectable units" determined for qualitative assays after limiting dilution.

Materials and Methods

Mycoplasma preparations

(a) Feasibility Study Panel

Strains of four mycoplasma species representing different phylogenetic branches within the bacterial class Mollicutes were selected for inclusion into the feasibility study panels:

Mycoplasma fermentans (PG18^T, NCTC 10117)
Mycoplasma orale (CH19299^T, NCTC 10112)
Mycoplasma pneumoniae (FH^T, NCTC 10119)
Acholeplasma laidlawii (PG8^T, NCTC 10116)

For cultivation of the mycoplasma strains Mycosafe Friis medium (Mycosafe Diagnostics GmbH, Vienna, Austria) was used which had been tested negative for mycoplasma DNA. For the feasibility study panel, the different strains were grown to an approximate target concentration of 10^5 colony forming units (CFU) per ml (*M. fermentans*, *M. orale* and *M. pneumoniae*) or 10^7 CFU/ml (*A. laidlawii*) and harvested during the exponential growth phase. An aliquot of each preparation was additionally diluted 1:100 using Mycosafe Friis medium as diluent. The actual number of CFU was determined for both the neat and the 1:100 diluted preparations (Table 1a). Species identity was confirmed by 16S rDNA sequence analysis and strain identity verified by Randomly Amplified Polymorphic DNA (RAPD) PCR analysis. The number of CFU was determined only for the characterization of the source materials. However, the correlation between CFU and nucleic acid concentration may depend on various factors, including mycoplasma species, strain, growth conditions, proportion of dead cells and the assays for CFU and nucleic acid determination.

(b) WHO IS candidate

For manufacture of the WHO IS candidate preparation *Mycoplasma fermentans* (PG18^T, NCTC 10117) was selected and grown to an approximate target concentration of 10^5 colony forming units (CFU) per ml and harvested during the exponential growth phase. Species identity was confirmed by 16S rDNA sequence analysis and strain identity verified by RAPD PCR analysis. The number of actual CFU/ml in the preparation was determined (Table 1b).

Lyophilization

For the feasibility study panel members, both the neat and the 1:100 diluted preparations were kept frozen at -60°C until freeze-drying (lyophilization) which was performed by a EN ISO 13485 certified company in Switzerland. Materials were thawed and, with gentle stirring, aliquoted as 0.5 ml volumes into 3 ml screw cap glass vials and immediately frozen. The coefficient of variation (CV) of filling was 1.2 %, as calculated by weight determination. Lyophilization was performed for 62 hours using the Christ Epsilon 2-25 instrument together with LPC-16/NT process documentation. Before sealing of the vials, the system was flooded with dry nitrogen to replace oxygen and moisture. The freeze-dried preparations were kept at -20°C until delivery to the feasibility study participants. Approximately 200 complete panels were produced in January 2012 for the feasibility study, with the exception of the *A. laidlawii* preparation with a target concentration of 10^7 CFU/ ml (Panel Member 9) where >3,000 vials were prepared.

The lyophilization protocol, used for the manufacture of feasibility study panels, was used to prepare the candidate WHO IS (*M. fermentans*). The coefficient of variation (CV) of filling was 1.0 %. The lyophilized candidate WHO IS preparation (PEI code number 8293/13) was prepared in January 2013 in a quantity of 5,126 vials.

All vials of lyophilized materials are stored at PEI at -20 °C with regular temperature monitoring. All manufacturing and monitoring records are held at PEI and are available on request by the ECBS.

Post-lyophilization investigations

A number of investigations were performed to characterize the lyophilized materials.

DNA detection after lyophilization

The potential impact of lyophilization on the detectability of mycoplasma DNA was addressed by comparative evaluation of lyophilized specimens (feasibility study panel members, candidate WHO IS) and their corresponding liquid source materials which had been kept frozen at -80°C.

By using three quantitative NAT assays of different design (Venor GeM qEP, Intego Mycoplasma (both Minerva Biolabs GmbH, Berlin, Germany) and MycoSEQ Mycoplasma Real-Time PCR (Applied Biosystems GmbH, Weiterstadt, Germany)) there was no significant difference in DNA detection determined between non-lyophilized (liquid) and lyophilized mycoplasma materials. Table 2 shows the results obtained with Intego Mycoplasma as a representative NAT assay.

Sterility tests

Sterility tests performed under aerobic and anaerobic conditions and using both liquid and solid media indicated no presence of viable bacteria in the reconstituted preparations

other than the chosen mycoplasma species, despite some manufacture steps (e.g. aliquoting) having been performed under non-aseptic conditions.

CFU after lyophilization

Post-lyophilization viability of the different mycoplasma preparations was determined for the lyophilized specimens and their direct source materials (including the additional freeze/thaw cycle). Viability of mycoplasma was reduced by freeze drying by a factor of approximately 0.2 $\log_{10}$ (*M. orale*), 1 $\log_{10}$ (*M. fermentans, M. pneumoniae*) and 2 $\log_{10}$ (*A. laidlawii*) (Table 3).

Residual moisture

The residual moisture in the freeze-dried samples was determined for a representative number of vials using an accredited method according to the European Pharmacopoeia. Residual moisture never exceeded 2% (0.6% - 1.9%) which is compliant with the "WHO recommendations for the preparation, characterization and establishment of international and other biological reference standards".[3]

Stability testing

An accelerated stability test programme was initiated simulating storage / transport temperatures of -20°C, 4°C, 23°C, 37°C and 45°C. The potential degradation of mycoplasma DNA under elevated temperature was determined at regular intervals using two quantitative NAT assays (Venor GeM qEP, Intego Mycoplasma). Over a period of 52 weeks no degradation of mycoplasma DNA for the *A. laidlawii* (code no. 9) preparation was observed, even at elevated temperatures (Figure 2); this is also the case for the *M. fermentans* candidate WHO IS preparation after 24 weeks of incubation. The stability studies are being continued.

Design of feasibility study

For the feasibility study two different panels of mycoplasma species were designed for either (semi)quantitative or qualitative NAT assays. Both panels contained the four selected species represented by panel members common between the two approaches. All panel members were coded.

Proposed study protocols differed between the two approaches. Users of (semi)quantitative assay were asked to perform one dilution step (1:10) for the high concentration panel members and to report numerical results, e.g. "copies/ml" or C_T-values. Users of qualitative assays were asked to perform end point dilutions and to report back positive or negative results obtained for the respective dilutions. Reconstitution of panel members was performed with 0.5 ml H_2O; all dilutions were to be performed with the diluent representing the usual negative test matrix of the

laboratory. Therefore a variety of different diluents was used including isotonic buffers, saline, culture medium, cultured cells, cell culture supernatant, virus bulk harvest or just H_2O.
All participants received detailed and specific study protocols and tables for result reporting attached to this report (Attachment 1a: Study protocol for (semi)quantitative NAT assays; Attachment 1b: Study protocol for qualitative NAT assays).
See Figure 3 for graphical presentation of the panels.

a) Panel for (semi)quantitative NAT assays

For (semi)quantitative NAT assays, the feasibility study panel consisted of 9 coded members representing lyophilized preparations of mycoplasma species of different concentrations and a medium control. The concentrations are indicated as the target CFU/ml before lyophilization.

Code no. 1: *A. laidlawii*, 10^5 CFU/ml
Code no. 2: *M. fermentans*, 10^3 CFU /ml
Code no. 3: *M. orale*, 10^3 CFU /ml
Code no. 4: *M. pneumoniae*, 10^3 CFU /ml
Code no. 5: negative (Friis medium)
Code no. 6: *M. pneumoniae*, 10^5 CFU /ml
Code no. 7: *M. orale*, 10^5 CFU /ml
Code no. 8: *M. fermentans*, 10^5 CFU /ml
Code no. 9: *A. laidlawii*, 10^7 CFU /ml

Each participant received three identical panels for three separate test runs. Participants were asked to test all preparations (1-9) neat and the preparations coded 6-9 additionally as 1:10 dilution, using the usual laboratory diluent as the matrix. Following this protocol, each participant performed 3 separate runs, resulting in at least 3 results per panel member and per 1:10 dilution, where proposed.

b) Panel for qualitative NAT assays

For qualitative NAT assays a feasibility study panel was designed which consisted of lyophilized preparations, coded as follows:

Code no. 1: *A. laidlawii*, 10^5 CFU/ml
Code no. 2: *M. fermentans*, 10^3 CFU/ml
Code no. 3: *M. orale*, 10^3 CFU/ml
Code no. 4: *M. pneumoniae*, 10^3 CFU/ml
Code no. 9: *A. laidlawii*, 10^7 CFU/ml

Each participant received three identical panels for three test runs. In the first test run, participants were asked to test the coded preparations neat and in $\log_{10}$-dilution series until test results became negative in order to determine the preliminary end point dilution for each panel member (lowest concentration tested as positive). For the subsequent two test runs, five half $\log_{10}$ dilutions around the pre-determined end point were proposed.

Design of comparability study

For comparison of the candidate WHO IS preparation with the *M. fermentans* preparation included in the feasibility study panel (Panel member 8) users of selected (semi)quantitative NAT assays were asked to determine the relative concentrations by testing replicates of both preparations neat and as 1:10 dilution and to report back either copies/ml or C_T-values; selected users of qualitative NAT assays were asked to test in replicates end-point dilution series of the two preparations, following a protocol analogous to the feasibility study.

Statistical methods

The statistical analysis was performed with SAS®/STAT software, version 9.3, SAS System for Windows. Estimation of end-point dilution and relative potency were determined using CombiStats Software, version 5.0, Release 2013, EDQM / Council of Europe.

Relative potencies

Evaluation of quantitative assays was performed without removing any outlying data. Assays giving C_T values and those reporting copies were evaluated separately. Potencies of samples were estimated relative to the Panel Member 8 (*M. fermentans*; neat material) or Panel Member 2 (*M. fermentans*; 1:100 dilution) with an assigned arbitrary value of 5.00 $\log_{10}$ International Units/ml (IU/ml) or 3.00 $\log_{10}$ International Units/ml (IU/ml), respectively, by parallel line assay on log transformed data (quantitative protocol) or probit transformed data (end-point dilution protocol).

Absolute potencies

Quantitative assays

Evaluation of the results reported by quantitative assays was restricted to dilutions in the range where the assays produce comparable data (linear range). For comparison of laboratories, the replicate results of each laboratory, corrected for the dilution factor, were combined as arithmetic mean of $\log_{10}$ copies/ml. Furthermore these estimates were combined to obtain an overall estimation for each sample.

Qualitative assays: End-point dilution procedure

The results from the independent runs were pooled to give a series of number positive out of number tested at each dilution. The pooled results of the single assays were evaluated by probit analysis to estimate the concentration at which 63% of the samples tested were positive (i.e. the dilution at which on average one single copy per sample tested could be expected under the assumption of an underlying Poisson distribution). The calculated end-point was used to give estimates expressed in NAT detectable units/ml after correcting for an equivalent volume of the test sample. The correction included volume extracted, volume eluted and fraction used for amplification.

Results

(a) Feasibility study

The feasibility study was announced in 2011 on the homepage of the "International Organization for Mycoplasmology" (IOM), in the "PDA Letter" (September 2011), by communication at various meetings and within the WHO Collaborating Centers network. Interest in voluntary participation was received from numerous organizations representing governmental authorities, biopharmaceutical manufacturers, IVD manufacturers and contract test services. There was no pre-selection of laboratories or NAT assays; all laboratories that expressed a willingness to participate were included. After resolution of various issues with customs and safety regulations in some countries, panels were dispatched at ambient temperature between May and August 2012 to the participating organizations.

Feasibility study panels, for evaluation in a total 25 (semi-)quantitative or qualitative mycoplasma NAT assays, were provided to 20 participating organizations.

The participants' names and organizations are provided in alphabetical order in Table 4. A set of panels for qualitative mycoplasma NAT was sent to one applicant who has not yet provided feedback (not included in Table 4). Most participants only performed one assay.

The amplification/detection systems and extraction procedures used by participants are listed in Table 5. There were few assays performed by more than one participant. The numbers representing commercially available tests are indicated, with the agreement of the manufacturers.

All assays of this evaluation targeted mycoplasma DNA, with the exception of two assays targeting mycoplasma RNA. However, results reported back for one of the RNA detection assays were incomplete and did not allow statistical evaluation, therefore this assay is not included further in this study report. The remaining qualitative assay for mycoplasma RNA detection was included in the feasibility study at a later stage (MilliPROBE; assay 26). The data obtained with this RNA assay were not included in

relative and absolute potency calculations which were restricted exclusively to assays detecting mycoplasma DNA.
NAT assays for mycoplasma DNA detection were numbered 1-25 (1–16 (semi-) quantitative; 17-25 qualitative), not reflecting the order in Tables 4 or 5.

Two of these assays were able to differentiate mycoplasma species (PLEX-ID Mycoplasma Detection assay, Abbott; CytoInspect PCR/Microarray, Greiner Bio-One). Both assays correctly identified the mycoplasma species of the different panel members.

There were no false-positive results obtained with the negative specimen (Panel Member 5) included in the panel for (semi-)quantitative assays, with exception of one in-house RT-PCR (assay 13) reporting high C_T-values for this panel member.

All assays were able to detect the four mycoplasma species included in the panels, with the exception of a (semi-)quantitative assay designed for detection of *M. pneumoniae* exclusively which did not cross react with the other species (assay 6) and two qualitative assays which failed to amplify either *M. orale* (assay 20) or *A. laidlawii* and *M. pneumoniae* (assay 22). These assays were excluded from statistical evaluation for the respective species.

The results reported by quantitative assays for the same panel member (e.g. in "copies/ml") differed between assays by a factor of up to 5 $\log_{10}$. The distribution of concentrations reported for neat panel members of the four mycoplasma species are shown in Figure 5.

The results reported for qualitative assays show a difference in sensitivity of the diluted samples between assays of up to 2 $\log_{10}$ for most assays and strains, with exceptions already mentioned. Figure 6 illustrates reactivity of individual qualitative assays (assay numbers 17 – 26) with replicate testing of the respective dilution series, providing for each assay either consistently positive or negative results (Figure 6, black or white boxes) or inconsistent results (Figure 6, grey boxes).

Statistical evaluation of feasibility study results

(a) (semi)quantitative NAT assays

The design of panel members combined with the proposed test protocol facilitated results for three different (by $\log_{10}$) concentrations ("high", "medium", "low") for each of the four mycoplasma species included in the panel: The panel itself included two preparations with 100-fold difference ("high", "low"), with the "high" concentration also being 1:10 diluted by the participant to the "medium" concentration.

The relative potency (relative to a "reference preparation" assigned with an arbitrary potency) determined for the residual panel members by the individual NAT assay was calculated. For this calculation either the "copies/ml" (reported by quantitative assays) or the C_T values reported by semi-quantitative real-time NATs were taken. For the statistical evaluation geometric mean values obtained for three individual panels in three test runs were used.

Overall, there was a high level of reproducibility between different test runs of an assay (intra-laboratory variability) with only a few outlier results excluded.

Estimation of relative potencies was done by means of a parallel line model with the validity preconditions of: (1) linearity within a dilution series for a mycoplasma species and (2) parallelism between the results obtained for different mycoplasma species (Table 6a). The linearity is necessary for potency calculation covering at least three concentration levels per species, and the parallelism (similar slopes) confirms that amplification efficiency of a NAT system is equivalent between the different mycoplasma species.

If both preconditions are fulfilled for two different mycoplasma species, the relative potency between these species may be calculated, as reported by the specific NAT assay (Table 6a). For the vast majority of assays, and mycoplasma preparations, these preconditions were fulfilled.

Figure 4 shows a graphical representation of quantitative ("copies/ml") and/or semi-quantitative ("C_T") results for the three $\log_{10}$ dilutions (1:1, 1:10, 1:100) for each assay. As an example, it becomes obvious that assays 3, 4 and 8 are non-linear within the *A. laidlawii* dilutions, but linear with the three other mycoplasma species.

(b) qualitative NAT assays

The data obtained by a NAT assay in three different dilution series were pooled to give a number positive out of number tested, at each dilution. As for (semi)quantitative assays, relative potencies of individual panel members were estimated relative to preparations included in the panel used as reference, e.g. *M. fermentans* (Table 6b). This estimation was performed by parallel line assay on probit transformed data using CombiStats Software, version 4.0, Release 2008, EDQM / Council of Europe.

Harmonization of mycoplasma NATs

In the relative potency calculation, a mycoplasma preparation may be defined as a "common calibrator", assigned an arbitrary unitage (e.g. candidate IU/ml), and the relative potency may be calculated for the other mycoplasma preparations in candidate IU/ml, too. The overall distribution of results expressed in candidate IU/ml by the different assays will reveal if the use of a "common calibrator" helps to harmonize results compared to analysis where no common calibrator is used, e.g. "copies/ml".

Harmonization is reflected by reduction of inter-assay or inter-laboratory variation. Respective calculations were performed using *A. laidlawii, M. orale* or *M. fermentans* as candidate calibrators. With *A. laidlawii* (preparation 9) a harmonization of NAT results was obtained, when compared to the distribution of "copies/ml" reported by the assays. However, the *A. laidlawii* preparations were not consistently picked up by all assays and different concentration levels were non-linear for some assays. Furthermore, compared to *M. fermentans*, the results appeared less consistent for *A. laidlawii* between runs of some assays.
M. orale was missed or underestimated by some assays, and *M. pneumoniae*, as an agent with higher biological hazard classification, would cause logistical shipment problems. In contrast, if the *M. fermentans* preparation as common calibrator (Panel Member 8, assigned arbitrary concentration of 10^5 candidate IU mycoplasma DNA/ml) the harmonization in candidate units reported for the other panel members was striking, especially for *M. orale* or *M. pneumoniae*, slightly less for *A. laidlawii* (Figure 7). The results reconfirm the lower consistency of relative results reported for the *A. laidlawii* preparations. A potential reason might be the higher concentration of mycoplasma DNA in Panel Member 9 compared to Panel Member 8, requiring more dilution steps for the qualitative assay study compounded further by higher variation between assays, and, additionally, being closer to the upper end (less linear) of the "linear range" of (semi)quantitative NATs, again contributing to higher variation between assays.

Comments from feasibility study participants

Few specific comments were received on the feasibility study report:
After completion of the feasibility study Laboratory 13 recognized an underestimation by 0.5 – 1.0 $\log_{10}$ of the reported results due to suboptimal reference DNA preparation; Laboratory 15 identified a consistent overestimation of the reported results by 1.0 $\log_{10}$ due to use of an incorrect calculation formula. These comments are not reflected in the feasibility study results where all results were included as reported; however, the absolute potency estimates were performed without and with the respective correction (see below).

(b) Comparability Study

The outcome of the feasibility study was the selection of *M. fermentans* as the most suitable candidate for a WHO IS. This decision was confirmed by discussions within the PDA Mycoplasma Task Force and at different meetings (e.g. SoGAT Ljubljana) and presented to the ECBS in October 2012 in an update report for the whole project.
A new lyophilized *M. fermentans* preparation was manufactured in January 2013 using the identical protocol as for preparation of the feasibility study panels. The *M. fermentans* concentration of the candidate WHO IS was targeted in the range of Panel Member 8. The mycoplasma DNA concentration and the CFU/ml were slightly lower both in the source material used for the candidate WHO IS and in the lyophilized materials when compared to the respective materials of Panel Member 8 (Tables 2b, 3b).
Several NAT assays, shown to be proficient in the feasibility study, were used to compare the two lyophilized preparations. In total, the two materials were compared by four different quantitative assays reporting results in copies/ml, by two semi-quantitative assays generating C_T values and by five qualitative assays in regard to potency differences. In all assays, the mycoplasma DNA content in the candidate WHO IS was determined to be slightly lower compared to Panel Member 8. The weighted mean difference factor was 0.316 (Table 7).

Proposed unitage for the WHO IS

Analytes in biological materials are often not traceable to SI units, e.g. mol or gram. In such cases WHO ISs are assigned an arbitrary unitage, the International Unit (IU). For NAT ISs developed for infectious diseases, generally the IU has been as closely aligned to the nucleic acid copy (e.g. genome copy) or to the NAT-detectable unit as possible. Therefore the mean "copies" reported by different quantitative assays for the feasibility study panel members were combined with the corresponding mean of "NAT detectable units" calculated from different qualitative assays' end point dilution results. For this calculation, the results of the feasibility study were used. The absolute potencies for the neat materials of the feasibility study are shown in Table 8 and the mean values in Table 9. The distribution of absolute potencies, calculated for qualitative and quantitative assays, is illustrated in Figure 8.
Calculations were performed without and with using the corrected values for assays 13 and 15; however, there is only a slight difference in the mean potency values of the different materials after correction for laboratories 13 and 15. The corrected mean value of the *M. fermentans* concentration in Panel Member 8 is 5.76 log copies/NAT detectable units/ml (575,439 copies/NAT detectable units/ml). The weighted mean difference between the candidate WHO IS and Panel Member 8 is factor 0.316, as determined in the comparability study (Table 7), resulting in 181,838 copies/NAT detectable units/ml for the candidate WHO IS. It is proposed to assign a unitage of 2×10^5 IU/ml to the candidate WHO IS.

Discussion

The collaborative study for the establishment of a WHO IS for mycoplasma DNA consisted of two parts. In the first part, the feasibility study, it could be shown that most of the assays designed for generic detection are able to consistently detect the different mycoplasma species provided in the panel. In the study there was only one assay which was designed for the specific detection of *M. pneumoniae* (Assay 6) which did not cross-react with the other mycoplasma species. The majority of assays of generic design detected the four mycoplasma species contained in the panel. Few in-house developed qualitative assays missed the one or other mycoplasma species, or showed quite different sensitivity limits between different species. In this study the need for an international reference preparation was confirmed by the high variation of quantitative assay results for the same preparations, best explained by lack of standardization of assays. We performed different calculations assuming that individual panel members would be used as common calibrator. Harmonization of assays would have been achieved with any panel member, however, the *M. fermentans* preparation appeared to be the most suitable candidate. The effect of harmonization of assays by the *M. fermentans* preparation as common calibrator is obvious. In this study each participant used the routine diluent matrix; spiking of a common mycoplasma preparation (harvested in Friis medium) into different commonly used diluents appeared to have no detrimental effect.
Due to the complex composition of biological materials the target analyte e.g. the nucleic acid of a virus or an organism, has to be extracted and purified prior to analysis. This kind of analyte is often not traceable to SI units, e.g. gram or mol. WHO ISs are often assigned instead with IU/ml to have a common value for the content of the analyte. In the field of NAT assays it has been sensible that the "IU/ml" of the WHO ISs are in the similar range to the "copies/ml" reported by quantitative assays and the "PCR-detectable units/ml" calculated from qualitative assay results obtained by replicate limiting dilutions. In the comparability study the weighted mean difference factor between Panel Member 8 and the corresponding candidate WHO IS was calculated using a number of assays of different type (quantitative, semiquantitative, qualitative) and design. Based on this differential factor and the absolute mean potency of Panel Member 8 in the feasibility study a concentration of 2×10^5 IU/ml is proposed to be assigned to the WHO IS for mycoplasma DNA. This value is in the same range as the copies/ml both reported by different quantitative NAT assays (Table 7) and obtained by an NAT independent method for measuring genomic mycoplasma DNA in Panel Member 8 after staining with PicoGreen, a fluorochrome selectively binding to dsDNA (S. Czurda, data not shown). The candidate WHO IS is proposed to be associated with an unitage for mycoplasma DNA, not RNA. First, there was only one assay in the feasibility study delivering

complete results based on RNA detection. Results obtained for the assay 26 (MilliPROBE) are reported for information and, of course, were not included in the statistical evaluation of the DNA detecting assays. Second, mycoplasma RNA is a measurand potentially very different from mycoplasma genomic DNA. The composition and levels of different mycoplasma RNAs depend on the metabolic status of the mycoplasma cell (e.g. cultivation conditions) and test results may strongly differ between different target RNAs selected by the assay(s). Furthermore, there are additional results from the stability study that mycoplasma RNA in lyophilized specimens appears to be clearly less stable under accelerated and stress conditions when compared to mycoplasma DNA (T. Hämmerle, personal communication). An analogous observation had been made in the collaborative study for establishment of a WHO IS for *Plasmodium falciparum* NAT assays.[4] In conclusion, assays targeting (potentially different) mycoplasma RNAs may be much more difficult to standardize by a reference preparation of the chosen design.

Comments from participants

In July 2013 the collaborative study draft report was circulated among all participants of the feasibility and comparability study for comments and feedback. Minor changes were proposed which are now included in this version of the report. The feedback was unanimously supportive for the establishment of the 1st WHO IS for mycoplasma DNA and the proposed unitage. Several participants proposed as next step the establishment of a WHO International Reference Panel containing different mycoplasma species for NAT assays.

Acknowledgements

We thank all the participants in the feasibility and the comparability study who invested much time and efforts in this exercise. We thank the members of the PDA Mycoplasma NAT Task Force (B. Potts, K. Brorson, S. Deutschmann, T. Hämmerle, L. Mallet) for their continued support and advice. Last not least, we are grateful to the manufacturers of mycoplasma assays who provided kits free of charge for this study.

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Tables

Table 1: Colony forming units (CFU) in mycoplasma source materials prior to lyophilization

(a) Feasibility study panel members

Mollicutes species/strain	**Titer Total Cells (CFU/ml)**	
	Targeted Titer	**Actual Titer**
Acholeplasma laidlawii PG8^T, NCTC 10116	10^7	0.98×10^6
	10^5	8.59×10^4
Mycoplasma fermentans PG18^T, NCTC 10117	10^5	1.67×10^5
	10^3	1.75×10^3
Mycoplasma orale CH19299^T, NCTC 10112	10^5	0.84×10^5
	10^3	0.87×10^3
Mycoplasma pneumoniae FH^T, NCTC 10119	10^5	1.47×10^5
	10^3	1.47×10^3
Friis culture medium	0	0

(b) Candidate WHO IS

Mollicutes species/strain	**Titer Total Cells (CFU/ml)**	
	Targeted Titer	**Actual Titer**
Mycoplasma fermentans PG18^T, NCTC 10117	10^5	0.79×10^5

Table 2: Mycoplasma DNA quantification before/after lyophilization by NAT (Intego Mycoplasma, Minerva)

(a) Feasibility study panel members

Mollicutes species/strain		**"Copies/ml"**	
	Code	**Liquid**	**Lyophilized**
Acholeplasma laidlawii PG8^T, NCTC 10116	1	4.77E+05	4.08E+05
	9	7.02E+07	4.03E+07
Mycoplasma fermentans PG18^T, NCTC 10117	2	1.44E+04	1.71E+04
	8	2.62E+06	2.23E+06
Mycoplasma orale CH19299^T, NCTC 10112	3	1.02E+03	1.56E+03
	7	1.44E+05	2.37E+05
Mycoplasma pneumoniae FH^T, NCTC 10119	4	2.53E+03	5.90E+03
	6	1.99E+06	1.07E+06
Friis culture medium	5	0	0

(b) Candidate WHO IS

Mollicutes species/strain	**"Copies/ml"**	
	Liquid	**Lyophilized**
Mycoplasma fermentans PG18^T, NCTC 10117	1.24E+06	7.33E+05

Table 3: Mycoplasma CFU/ml determination before/after lyophilization

(a) Feasibility study panel members

Mollicutes species/strain		**CFU/ml**	
	Code	**Liquid**	**Lyophilized**
Acholeplasma laidlawii PG8^{T}, NCTC 10116	1	6.99E+04	6.90E+02
	9	3.33E+06	5.45E+04
Mycoplasma fermentans PG18^{T}, NCTC 10117	2	5.78E+02	9.25E+01
	8	6.95E+04	1.00E+04
Mycoplasma orale CH19299^{T}, NCTC 10112	3	4.57E+02	3.11E+02
	7	4.63E+04	2.44E+04
Mycoplasma pneumoniae FH^{T}, NCTC 10119	4	1.10E+03	1.98E+02
	6	1.15E+05	2.44E+04
Friis culture medium	5	0	0

(b) WHO IS candidate preparation

Mollicutes species/strain	**CFU/ml**	
	Liquid	**Lyophilized**
Mycoplasma fermentans PG18^{T}, NCTC 10117	7.54E+04	2.63E+03

Table 4: Feasibility study participants (in alphabetical order)

Name	Organization
Eric Abachin, Laurent Mallet	Sanofi Pasteur, Paris, France
Freek Blanken, Nigel Stapleton	Microsafe Laboratories, Leiden, NL
Francesca Bonci	Kedrion Biopharmaceuticals, Castelvecchio Pascoli, Italy
Susan Brand-Hoefs	Merck, Oss, Netherlands
Vicki Chalker	Health Protection Agency, London, UK
Stefan Czurda, Ursula Ulrych, Renate Rosengarten	Mycosafe Diagnostics GmbH, Vienna, Austria
Alena Dabrazhynetskaya, Vladimir Chizhikov	FDA/CBER/LMD, Kensington, USA
Thomas Hämmerle	Baxter AG, Orth/Donau, Austria
Michael Hantman	Charles River Biopharmac. Services, Malvern, USA
Matthias Hornschuh, Dirk Vollenbroich	Minerva Biolabs, Berlin, Germany
Holger Kavermann, Sven Deutschmann	Roche Diagnostics, Penzberg, Germany
Claudia König, Oliver Karo	Paul Ehrlich Institut, Langen, Germany
Fabrizio Lecce	Merck Serono, Ivrea, Italy
Andreas Lindauer	Synlab MVZ, Weiden, Germany
Cynthia Martino	Bionique Testing Laboratories, Saranac Lake, USA
Dietmar Mayer	IDT Biologika, Dessau, Germany
Alexandra Priessner, Michael Molitor	Novartis Vaccines and Diagnostics, Marburg, Germany
Brian Mondeja Rodriguez	Tropical Medicine Institute, Havana, Cuba
Walter Rudorfer, Joerg Stappert	Greiner bio-one, Frickenhausen, Germany
Rangarajan Sampath	IBIS Biosciences, Abbott, Carlsbad, USA
Yuko Sasaki	National Institute of Infectious Diseases, Tokyo, Japan

Table 5: NAT assays included in the feasibility study

Amplification / Detection	**Extraction**	**Assay No.**
NAT assays used for (semi)quantitative evaluation		
In-house RT-PCR 1	QIAamp Viral RNA Mini Kit	
In-house RT-PCR 2	DNeasy Blood & Tissue Kit	
In-house RT-PCR 3	QiaSymphony	
In-house RT-PCR 4	Phenol / Chloroform	
Intego Mycoplasma	InviMag Universal Kit/IG	4
Intego Mycoplasma	Chemagen RSMI	11
Intego Mycoplasma	QIAamp DNA Blood Mini Kit	15
Microsart AMP Mycoplasma	InviMag Universal Kit/IG	3
MycoSEQ Mycoplasma Real-Time PCR	PrepSEQ Sample Preparation Kit	5, 8, 9, 10
MycoSEQ Mycoplasma Real-Time PCR	NucliSENS easyMAG	7
MycoTool Mycoplasma RealTime PCR	MagNA Pure	1
PLEX-ID Mycoplasma Detection assay	Bead beating lysis	2
Venor GeM qEP	QIAamp DNA Blood Mini Kit	15
NAT assays used for qualitative evaluation		
CytoInspect PCR/Microarray	CytoInspect DNA Extraction Kit	18, 21, 24
In-house nested PCR	QIAamp DNA Mini Kit	
In-house RT-PCR 5	Phenol / Chloroform	
In-house RT-PCR 6	Silica columns	
MilliPROBE	Target capture (rRNA)	26
MycoTool Mycoplasma Amplif. and Det. Kits	Manual, 2-propanol	19
Venor GeM Advance	MB DNA Extraction Kit	20
Venor GeM Advance	QIAamp DNA Blood Mini Kit	25

Table 6: Potencies relative to Panel Member 8, *Mycoplasma fermentans*

(a) (Semi)quantitative NAT assays

For the sample data including at least 3 dilutions (1:1, 1:10, and 1:100), potencies were calculated relative to the reference preparation, *M. fermentans* (Panel Member 8), using a parallel line model. For the reference preparation an arbitrary potency of 5.0 $\log_{10}$ candidate IU/ml was used for the calculations.

	A. laidlawii			*M. orale*			*M. pneumoniae*				
Assay code	Relative potency	95%-Confidence Interval		Relative potency	95%-Confidence Interval		Relative potency	95%-Confidence Interval		Measured	Remark
1	7.59	7.31	7.92	5.08	4.88	5.28	5.41	5.20	5.62	C_T values	Borderline linearity[1]
2	5.87	5.45	6.35	4.94	4.45	5.45	6.18	5.71	6.77	copies	Borderline parallelism[2]
3				4.07	3.74	4.36	4.63	4.34	4.91	copies	*A. laidlawii* non-linear
3							4.66	4.53	4.79	C_T values	*A. laidl.* non-linear; *M. orale* non-parallel
4				4.12	3.91	4.31	4.75	4.56	4.93	copies	*A. laidlawii* non-linear; borderline linearity[1]
4							4.78	4.65	4.90	C_T values	*A. laidl.* non-linear; *M. orale* non-parallel
5	6.14	5.86	6.47				4.46	4.18	4.73	C_T values	Borderline linearity[1], *M. orale* non-linear
6										copies	Only 2 dilutions
6										C_T values	Only 2 dilutions
7	6.30	6.17	6.43	4.40	4.29	4.51				copies	Borderline linearity[1], *M. pneum.* non-lin.
8				4.54	3.44	5.43	4.54	3.44	5.43	C_T values	*A. laidlawii* non-linear
9	5.82	5.50	6.16	4.30	3.97	4.62	4.28	3.94	4.59	C_T values	
10										C_T values	Only 2 dilutions
11	7.18	6.96	7.41	4.03	3.84	4.20	4.47	4.30	4.63	C_T values	Borderline parallelism[2]
12										copies	Only 2 dilutions
13	6.59	6.43	6.76	4.25	4.10	4.39	4.74	4.60	4.88	copies	Borderline parallelism[2]
13				4.28	4.17	4.39	4.76	4.65	4.87	C_T values	*A. laidlawii* non-linear
14										copies	Only 2 dilutions
15	6.45	6.16	6.77	3.89	3.60	4.16	4.76	4.49	5.01	copies	
15	6.54	6.27	6.85	3.92	3.65	4.18	4.77	4.52	5.01	C_T values	
16	7.13	6.81	7.51	4.18	3.90	4.44	4.65	4.38	4.90	copies	
16	7.23	7.06	7.42	4.24	4.10	4.38	4.55	4.42	4.68	C_T values	Borderline parallelism[2]
Mean[3]	**6.62**	6.23 – 7.02		**4.30**	4.10 – 4.50		**4.77**	4.54 – 5.01			

1 – p-value for non-linearity between 0.01 and 0.05; 2 – p-value for non-parallelism between 0.01 and 0.05; 3 – Combined potency

(b) Qualitative NAT assays

For sample data including potencies were calculated relative to the reference preparation, *Mycoplasma fermentans*, using a parallel line assay on probit transformed data. For the *M. fermentans* reference preparation a potency of 3.0 $\log_{10}$ candidate IU/ml was used for the calculations.

	A. laidlawii 1:100			*M. orale* 1:100			*M. pneumoniae* 1:100			*A. laidlawii* 1:1			Remark
Lab code	Relative potency	95%-Confidence Interval		Relative potency	95%-Confidence Interval		Relative potency	95%-Confidence Interval		Relative potency	95%-Confidence Interval		
17	6.80	6.14	7.47	4.25	3.59	4.91	4.59	3.93	5.25	6.20	5.55	6.87	
18	5.56	4.78	6.27	4.83	4.06	5.60	4.57	3.80	5.29	4.97	4.21	5.69	
19	5.00	4.29	5.71	3.77	3.08	4.47	4.36	3.67	5.04	5.59	4.89	6.29	fixed slope
20	8.52	7.13	10.61				5.00	3.57	6.43	6.65	5.30	8.51	*M. orale* negative
21	6.26	5.41	7.17	5.02	4.19	5.88	5.58	4.76	6.42	5.41	4.59	6.23	
22				4.25	3.73	4.77							*A. laidlawii*, *M. pneumoniae* neg.
23	6.00	5.34	6.66	4.40	3.74	5.06	4.80	4.14	5.47	6.20	5.54	6.87	fixed slope
24	5.82	5.13	6.51	3.80	3.10	4.47	4.23	3.53	4.90	5.42	4.72	6.08	
25	7.53	7.10	8.02	4.16	3.66	4.56	4.85	4.41	5.28	7.49	7.06	7.90	
Mean[2]	**6.44**	5.48 – 7.39		**4.25**	4.03 – 4.47		**4.71**	4.48 – 4.95		**5.99**	5.31 – 6.67		

1 – Slope was not estimable and thus fixed to 0.869 (with 0.869 = 1/ln(3.16) and 3.16 as dilution step width) for potency estimation; 2 – Combined potency

Table 7
Relative potency of WHO IS versus Panel Member 8 (Reference)

Assay	Assay Type	Potency of WHO IS candidate relative to Panel Member 8	95% Confidence Interval		Copies/ml reported for WHO IS candidate
In-house 1	quantitative	**0.288**	0.163	0.477	7.4×10^5
In-house 2	quantitative	**0.272**	0.110	0.535	5.2×10^5
Microsart AMP Mycoplasma	quantitative	**0.282**	0.204	0.382	2.7×10^5
Intego Mycoplasma	quantitative	**0.309**	0.223	0.421	3.1×10^5
MycoTool Mycoplasma RealTime PCR	semi-quantitative	**0.270**	0.202	0.358	
MycoSEQ Mycoplasma Real-Time PCR	semi-quantitative	**0.392**	0.274	0.549	
In-house 3	qualitative	**0.150**	0.000	4.251	
In-house 4	qualitative	**0.750**	0.356	1.582	
CytoInspect PCR/Microarray	qualitative	**0.603**	0.062	5.707	
Venor GeM Advance	qualitative	**0.747**	0.204	2.658	
MycoTool Mycoplasma Amplification and Detection Kits	qualitative	**0.383**	0.140	1.049	
Combined[1]		**0.316**	0.277	0.360	

1 – Weighted combination estimator
Relative potencies were estimated by means of a parallel line model (quantitative data) and Probit analysis (qualitative data; Spearman-Kaerber method was used instead of Probit in cases, where the slope of the curves could not be estimated).
The overall potency estimator is based on a weighted combination of results.
No outliers were removed from combination due to relatively homogeneous results.

Table 8
Absolute Potency Estimates (Feasibility Study Panel Members)
as log copies/ml (quantitative assays; 2-16) or log NAT detectable units/ml (qualitative assays; 17-25). For assays 13 and 15 both originally reported (not corrected) and corrected (* *italic)* values are shown

	Acholeplasma laidlawii		
Assay	Estimate	95% Confidence Interval	
2	5.57	3.66	7.48
3	6.82	5.30	8.33
4	6.65	5.21	8.09
7	7.55	7.20	7.90
12	7.66	4.78	10.54
13	6.30	5.77	6.83
*13**	*6.80*	*6.27*	*7.33*
14	6.91	4.67	9.15
15	9.11	8.72	9.50
*15**	*8.11*	*7.72*	*8.50*
16	9.66	9.28	10.04
17	7.14	6.60	7.69
18	5.84	5.48	6.20
19	6.58	6.23	6.92
20	7.03	5.43	8.63
21	6.58	5.84	7.32
22			
23	6.24	5.91	6.57
24	6.97	6.64	7.31
25	7.30	7.09	7.51

	Mycoplasma fermentans		
Assay	Estimate	95% Confidence Interval	
2	5.09	3.97	6.21
3	6.02	5.62	6.42
4	5.90	5.48	6.31
7	6.12	5.57	6.68
12	5.94		
13	4.80	4.57	5.02
*13**	*5.30*	*5.07*	*5.52*

14	5.35	3.73	6.98
15	7.70	7.47	7.92
*15**	*6.70*	*6.47*	*6.92*
16	7.51	7.11	7.91
17	5.64	5.14	6.08
18	5.58	5.03	6.11
19	6.27	5.77	6.78
20	4.48	3.32	5.39
21	5.76	5.14	6.33
22	5.80	5.32	6.14
23	5.14	4.67	5.61
24	6.36	5.85	6.84
25	4.79	4.47	5.10

	Mycoplasma orale		
Assay	Estimate	95% Confidence Interval	
2	4.44	3.77	5.12
3	5.09	4.74	5.44
4	4.99	4.73	5.25
7	5.43	4.86	5.99
12	5.64		
13	4.09	4.00	4.18
*13**	*4.59*	*4.50*	*4.68*
14	4.94	3.02	6.86
15	6.62	6.31	6.93
*15**	*5.62*	*5.31*	*5.93*
16	6.68	6.35	7.01
17	4.89	4.39	5.34
18	5.40	4.85	5.96
19	5.05	4.57	5.53
20			
21	5.78	5.17	6.38
22	5.05	4.57	5.39
23	4.54	4.07	5.00
24	5.16	4.66	5.60
25	3.95	3.57	4.22

	Mycoplasma pneumoniae		
Assay	Estimate	95% Confidence Interval	
2	5.35	4.43	6.28
3	5.65	5.06	6.24
4	5.64	4.97	6.30
6	5.28	3.57	6.98
7	5.39	4.60	6.18
12	5.67		
13	4.55	4.33	4.78
*13**	*5.05*	*4.83*	*5.28*
14	5.07	2.99	7.15
15	7.46	7.04	7.88
*15**	*6.46*	*6.04*	*6.88*
16	7.15	6.93	7.37
17	5.23	4.73	5.67
18	5.14	4.60	5.63
19	5.63	5.17	6.10
20	4.48	3.32	5.39
21	6.34	5.74	6.91
22			
23	4.94	4.47	5.42
24	5.59	5.08	6.04
25	4.64	4.33	4.94

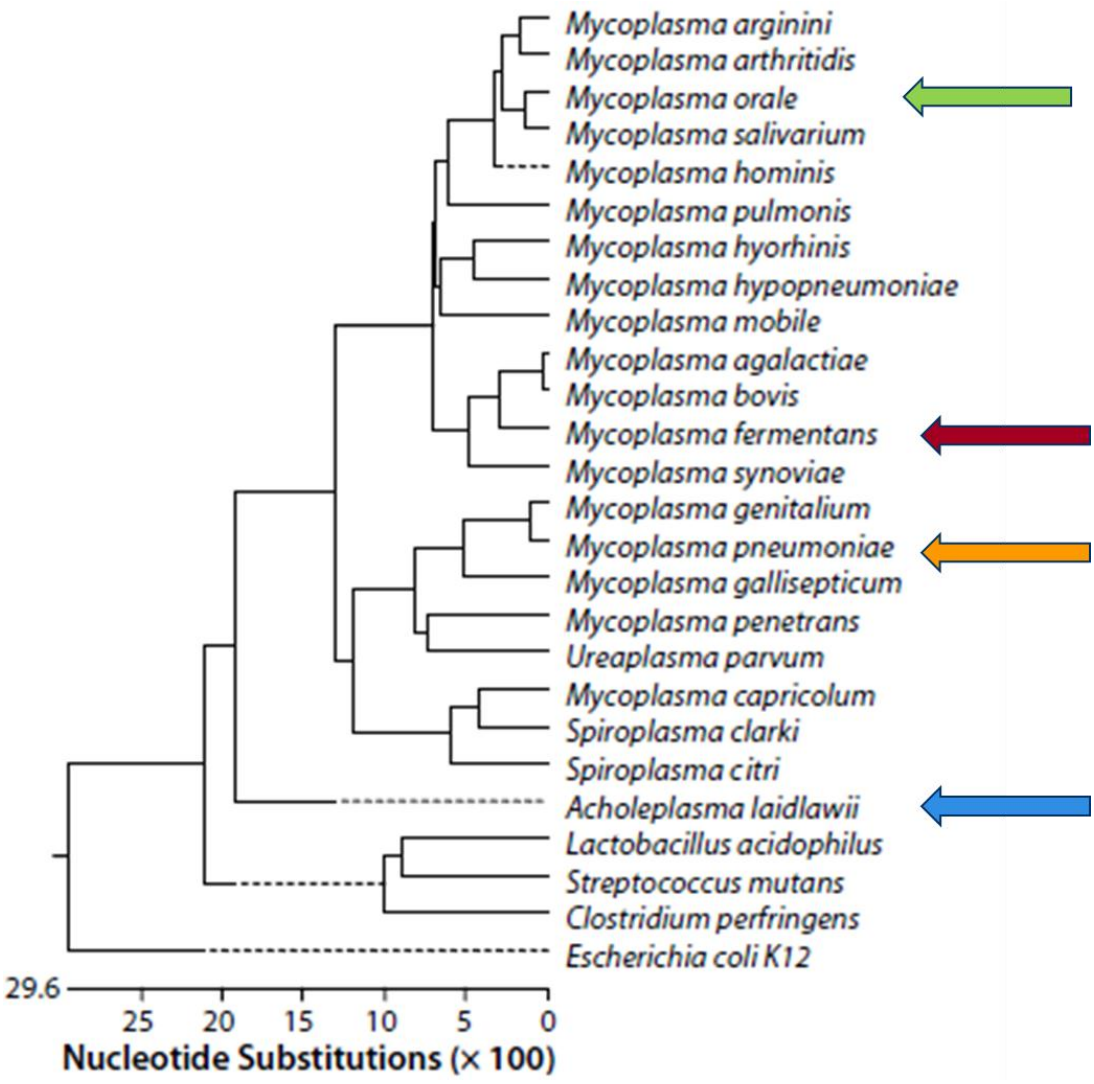
Table 9
Absolute Potency Estimates (Feasibility Study Panel Members)

Overall mean estimates with (bold) and without (in brackets) corrections for Lab 13 and 15

Sample	N (assays)	Mean Estimate	95% Confidence Interval	
Acholeplasma laidlawii	18	**7.02** (7.05)	**6.55** (6.52)	**7.50** (7.59)
Mycoplasma fermentans	19	**5.76** (5.79)	**5.41** (5.37)	**6.12** (6.21)
Mycoplasma orale	18	**5.13** (5.16)	**4.81** (4.78)	**5.45** (5.54)
Mycoplasma pneumoniae	19	**5.48** (5.51)	**5.16** (5.12)	**5.81** (5.91)

Figures

Figure 1
Phylogenetic Tree of Mycoplasma species



Arrows indicate the different species included into the feasibility study panels

Figure 2

Accelerated stability testing of lyophilized mycoplasma

(Intego, Minerva)

Acholeplasma laidlawii, Panel Member 9

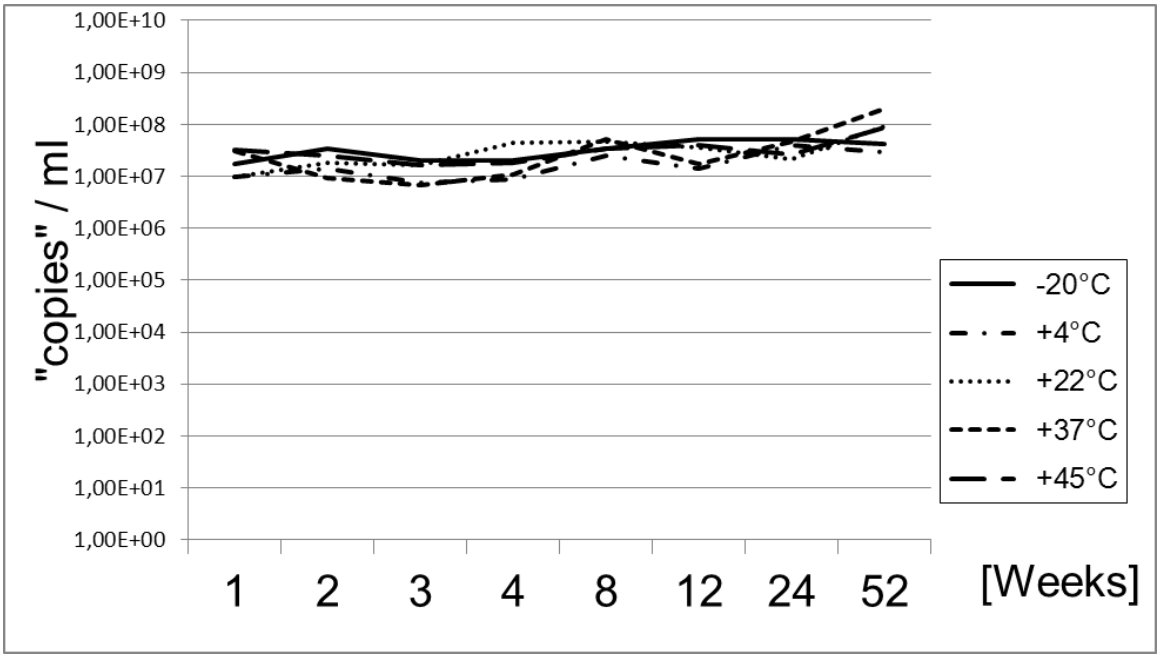
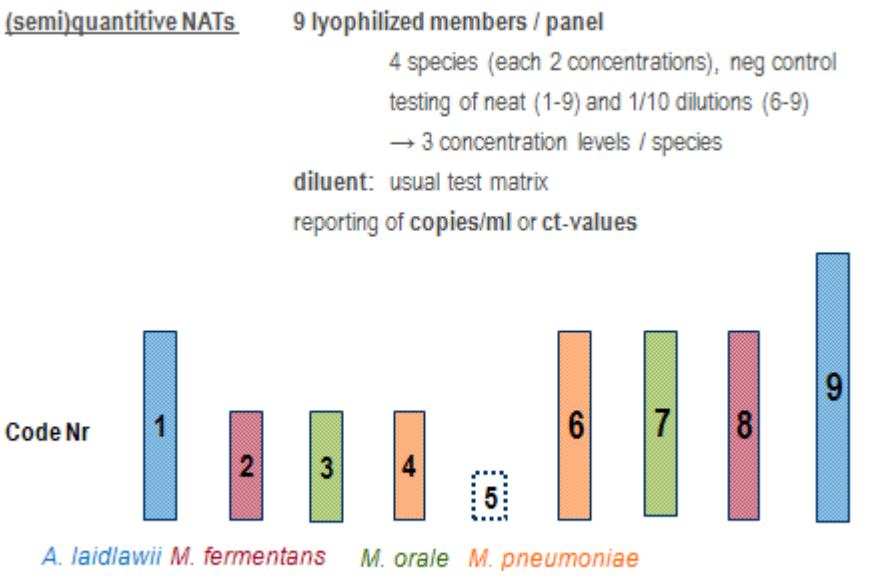


Figure 3
Composition of coded feasibility study panels

(a) Panel designed for (semi)quantitative mycoplasma NATs
(the size of the bars reflects approximate content of CFU (log_{10} scale))



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b) Panel designed for qualitative mycoplasma NATs
(the size of the bars reflects approximate content of CFU (log10 scale))

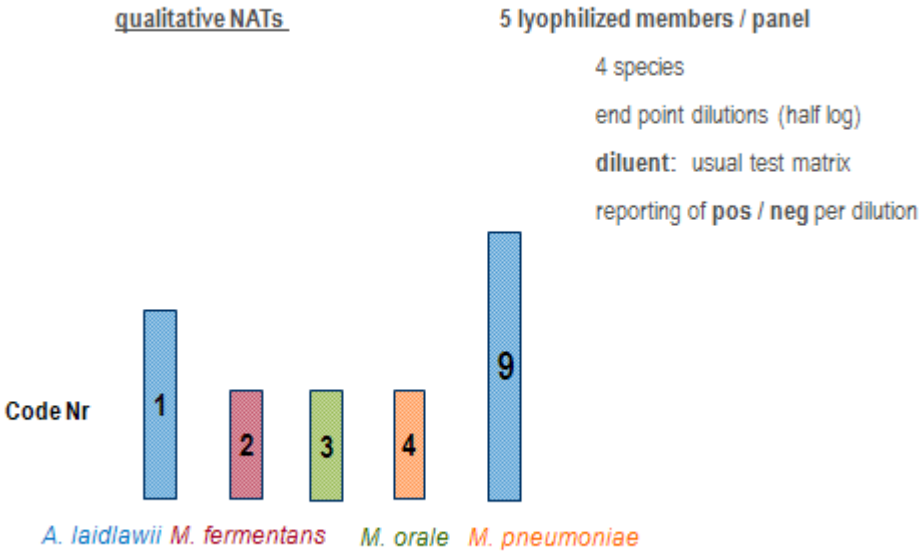

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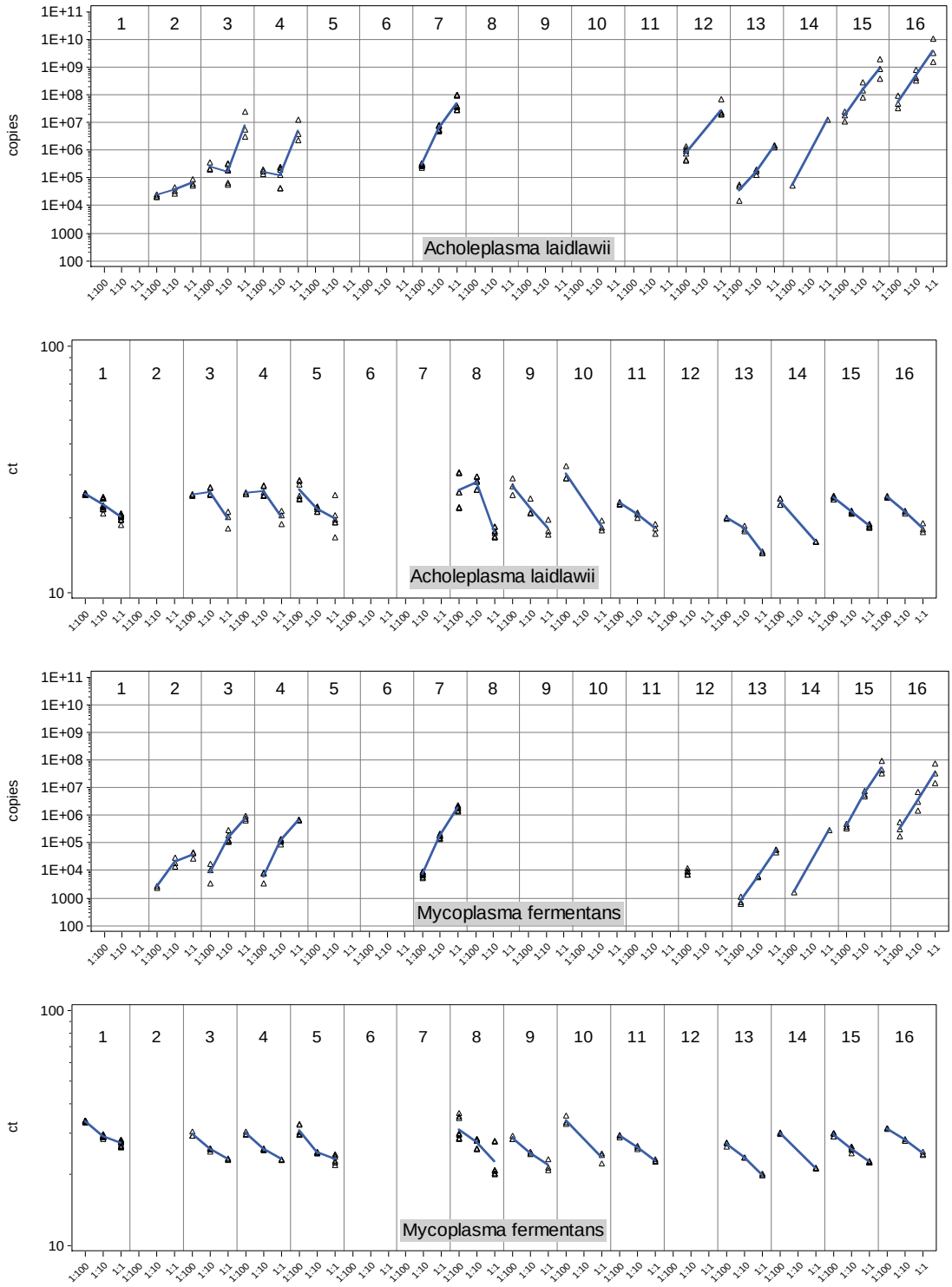
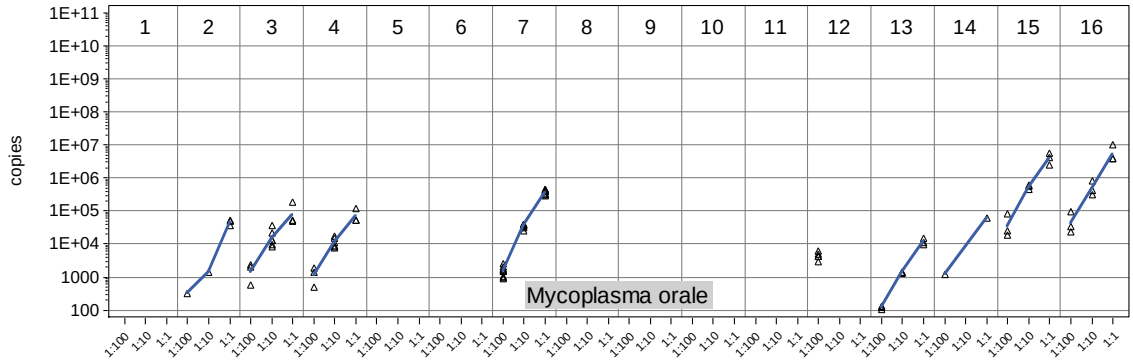
Figure 4

Result graphs for (semi)quantitative NAT assays (1-16) reported as "copies" or "C_T" for three concentration levels of individual Mycoplasma species

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1E+10
1E+09
1E+08
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1E+04
1000
100
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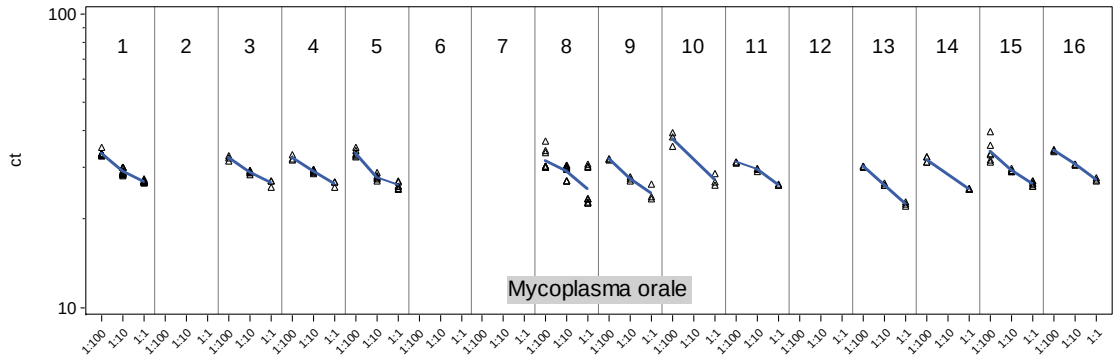
Mycoplasma orale
1:100 1:10 1:1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10
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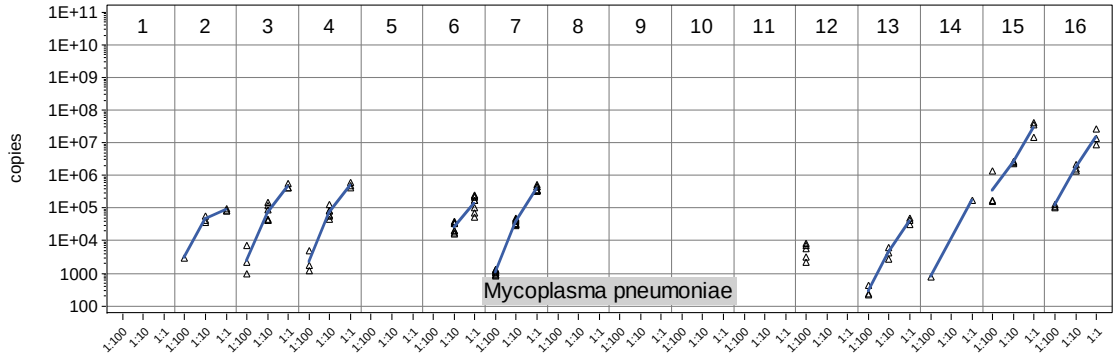
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Mycoplasma pneumoniae
1:100 1:10 1:1

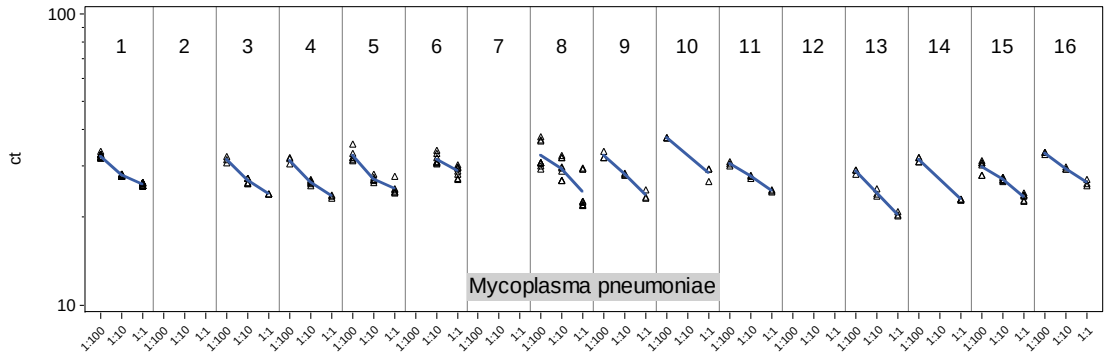
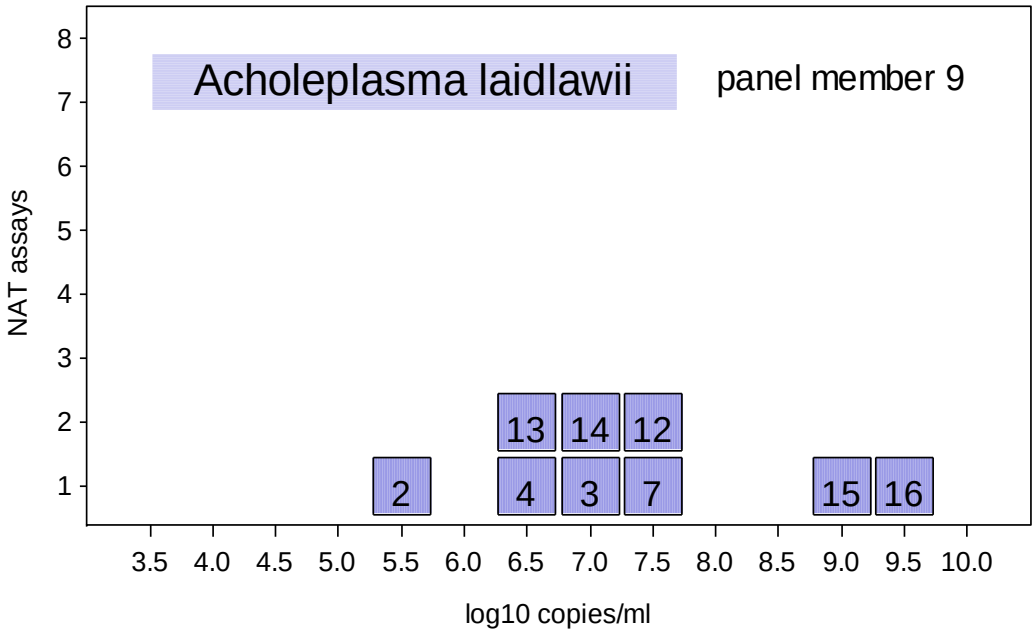
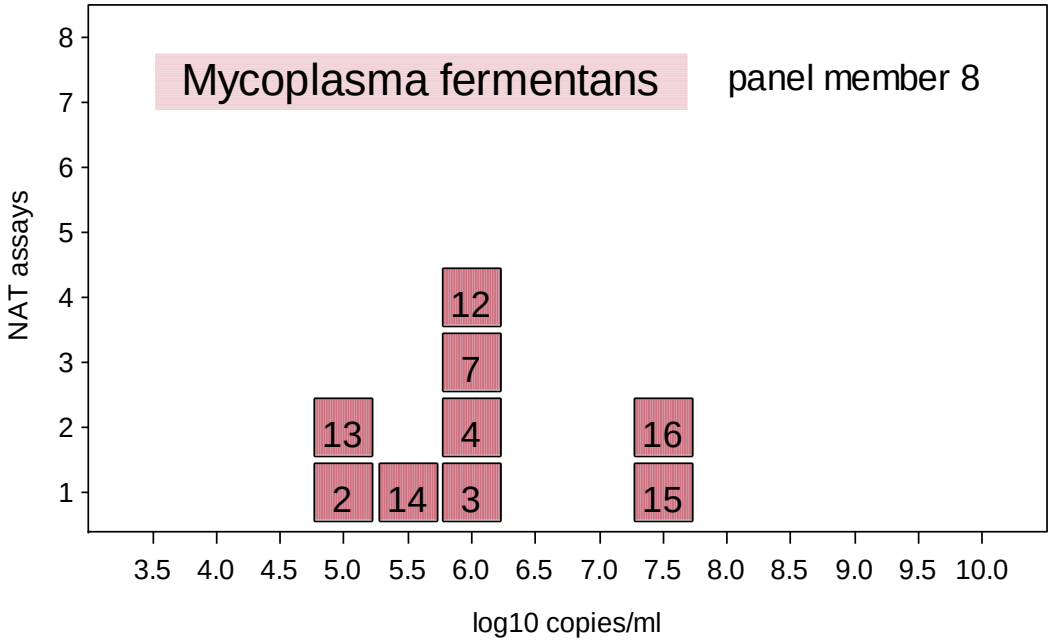
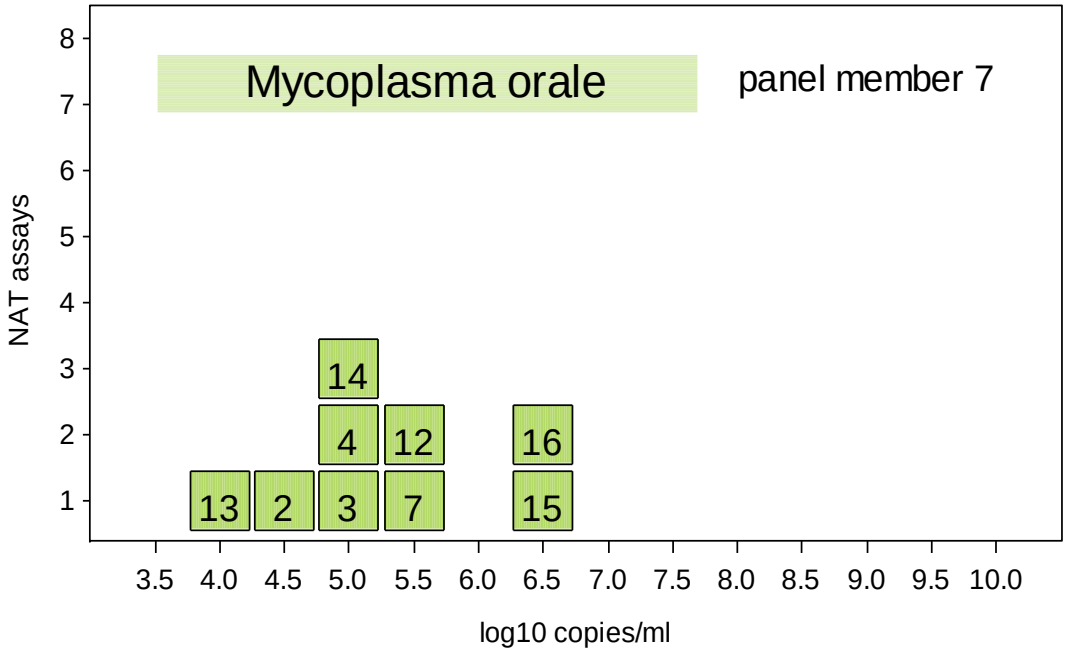

ct
100
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Mycoplasma pneumoniae
1:100 1:10 1:1

Figure 5
Distribution of "copies/ml" as reported by quantitative assays
Assay numbers are indicated in boxes, each box represents a different assay.






Mycoplasma orale
panel member 7
NAT assays
log10 copies/ml
13
2
3
7
4
12
14
16
15

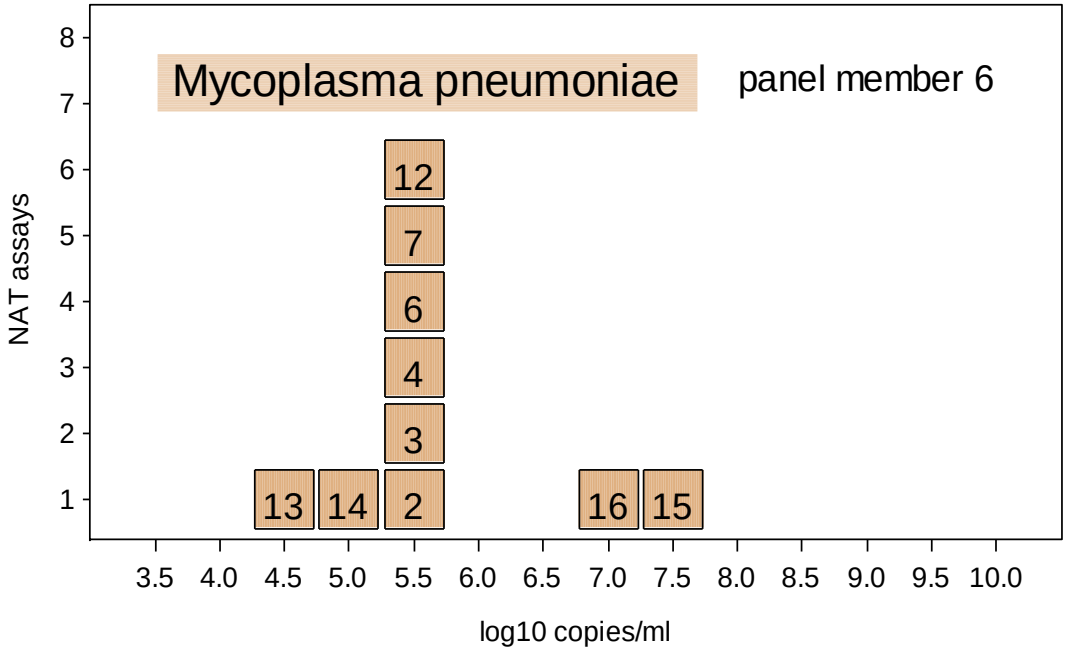

Mycoplasma pneumoniae
panel member 6
NAT assays
log10 copies/ml
13
14
2
3
4
6
7
12
16
15

Figure 6
Result graphs for qualitative NAT assays (17-26) reported for replicates of serial dilutions (neat to -6.5 log_{10}) of panel members

	Panel Member 1, *A. laidlawii*													
Assay No.														
	neat	-0.5	-1.0	-1.5	-2.0	-2.5	-3.0	-3.5	-4.0	-4.5	-5.0	-5.5	-6.0	-6.5
17														
18														
19														
20														
21														
22														
23														
24														
25														
26														

	Panel Member 2, *M. fermentans*													
Assay No.														
	neat	-0.5	-1.0	-1.5	-2.0	-2.5	-3.0	-3.5	-4.0	-4.5	-5.0	-5.5	-6.0	-6.5
17														
18														
19														
20														
21														
22														
23														
24														
25														
26														

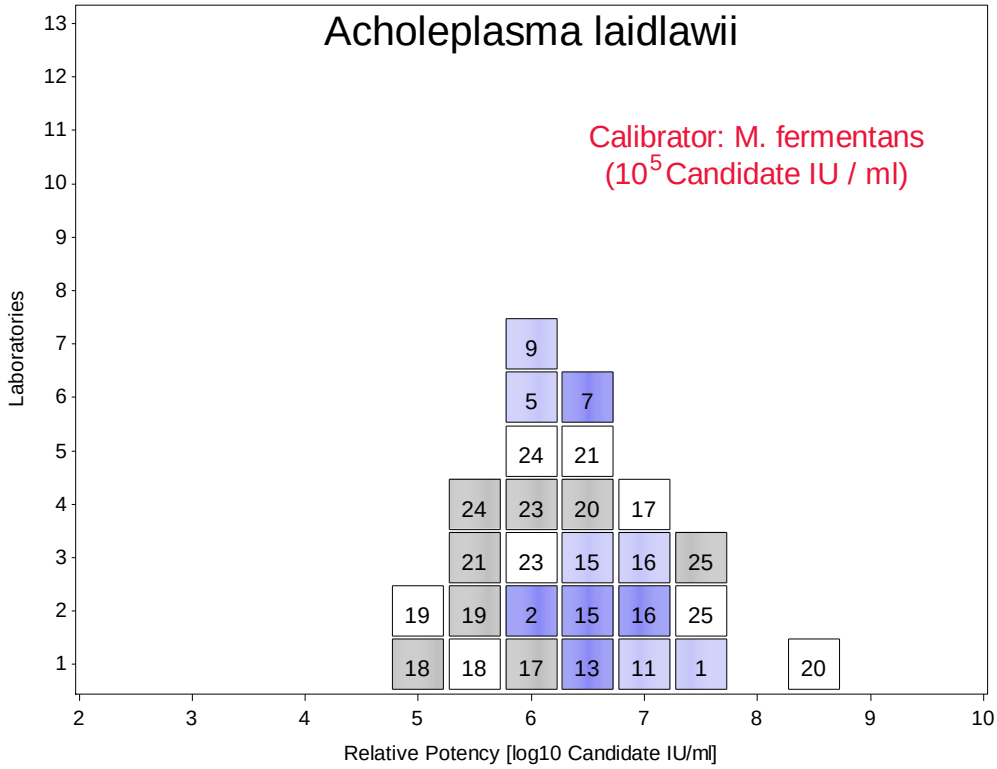
	Panel Member 3, *M. orale*													
Assay No.														
	neat	-0.5	-1.0	-1.5	-2.0	-2.5	-3.0	-3.5	-4.0	-4.5	-5.0	-5.5	-6.0	-6.5
17														
18														
19														
20														
21														
22														
23														
24														
25														
26														

	Panel Member 4, *M. pneumoniae*													
Assay No.														
	neat	-0.5	-1.0	-1.5	-2.0	-2.5	-3.0	-3.5	-4.0	-4.5	-5.0	-5.5	-6.0	-6.5
17														
18														
19														
20														
21														
22														
23														
24														
25														
26														

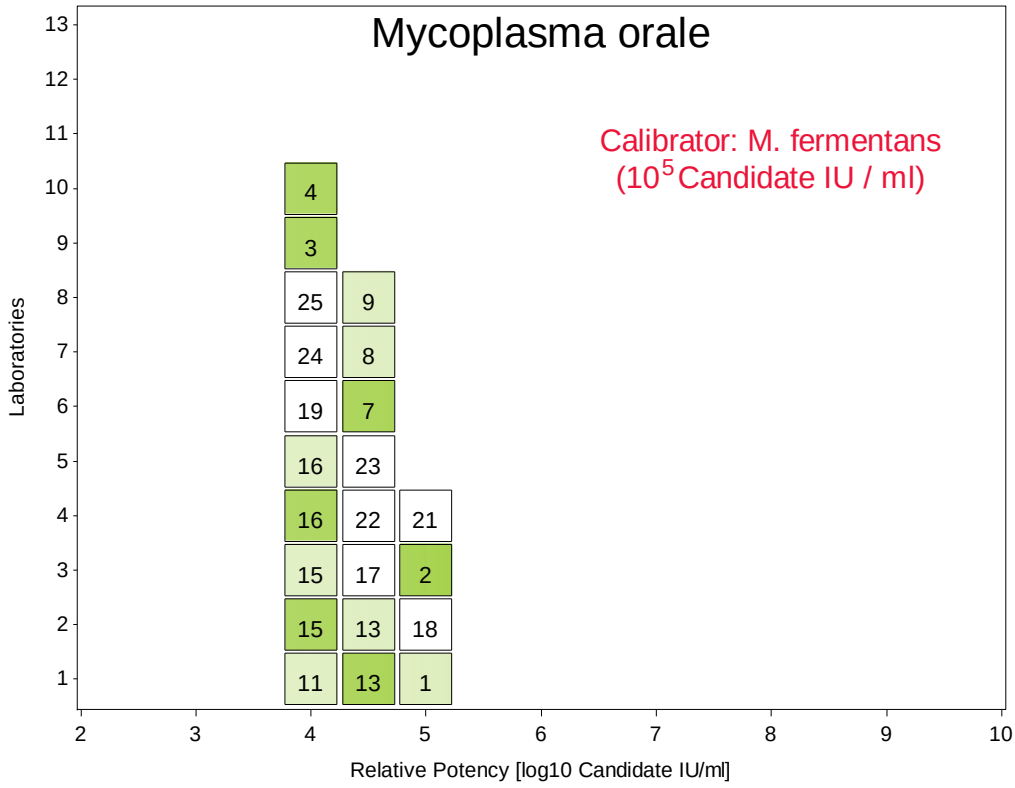
	Panel Member 9, *A. laidlawii*													
Assay No.														
	neat	-0.5	-1.0	-1.5	-2.0	-2.5	-3.0	-3.5	-4.0	-4.5	-5.0	-5.5	-6.0	-6.5
17														
18														
19														
20														
21														
22														
23														
24														
25														
26														

“consistently positive” (black), “consistently negative” (white) or “pos/neg” (grey)

Figure 7
Combined relative potency evaluation for quantitative and qualitative Assays (Reference candidate: *M. fermentans*)
Distribution of candidate IU/ml as calculated for (semi)quantitative assays (1-16; dark colour: calculation based on "copies"; light colour: calculation based on "C_T-values") and for qualitative assays (17-25) with using *M. fermentans* with an aribitrary concentration of 10^5 candidate IU/ml (Panel Member 8) or of 10^3 candidate IU/ml (Panel Member 2) as calibrator. The assay number is included in the box.



Qualitative assays: grey: for *A.laidlawii* 1:1 concentration (panel code 9); white: for *A. laidlawii* 1:100 concentration (panel code 1)


Mycoplasma orale
Calibrator: M. fermentans
(10^5 Candidate IU / ml)
Laboratories
Relative Potency [log10 Candidate IU/ml]
4
3
25 9
24 8
19 7
16 23
16 22 21
15 17 2
15 13 18
11 13 1

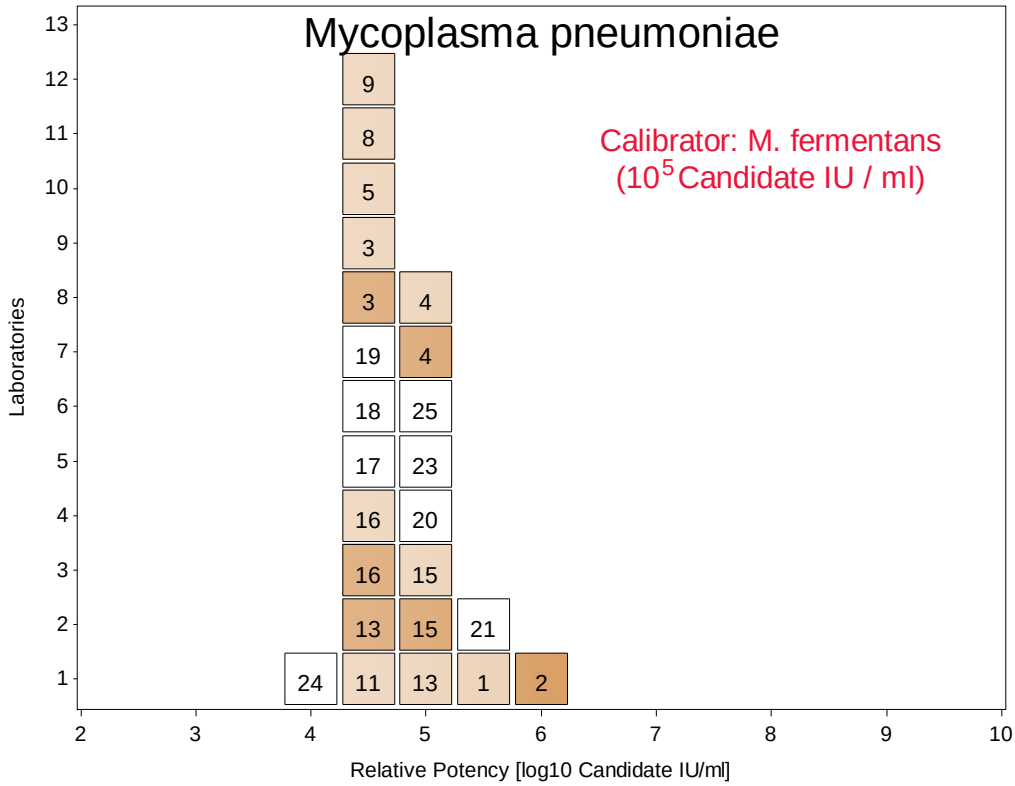
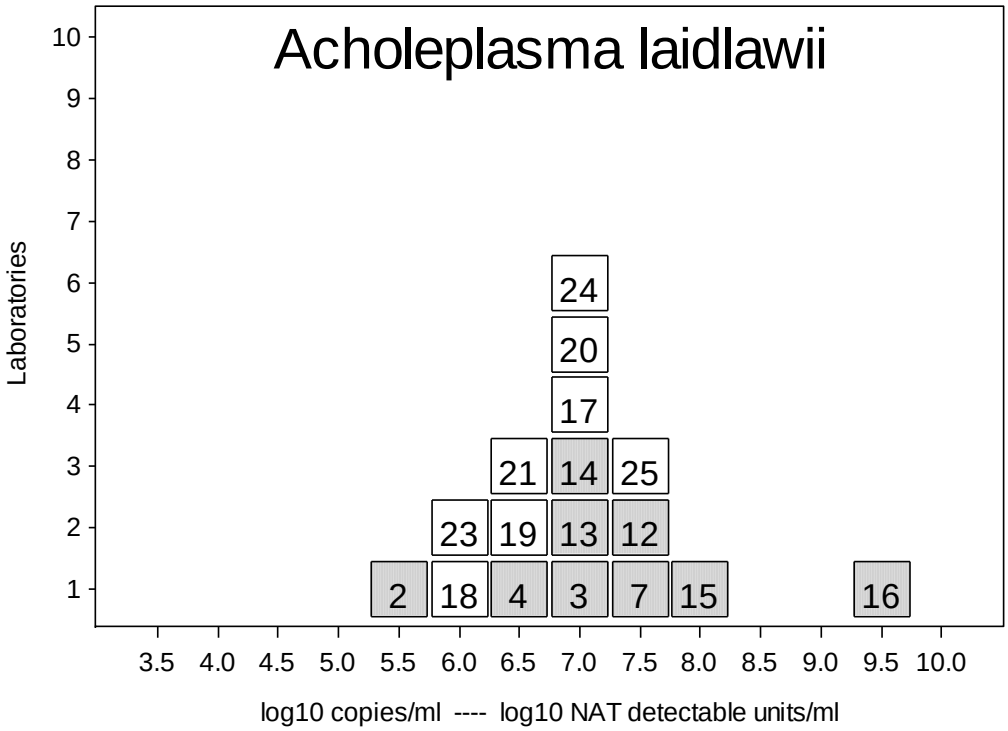
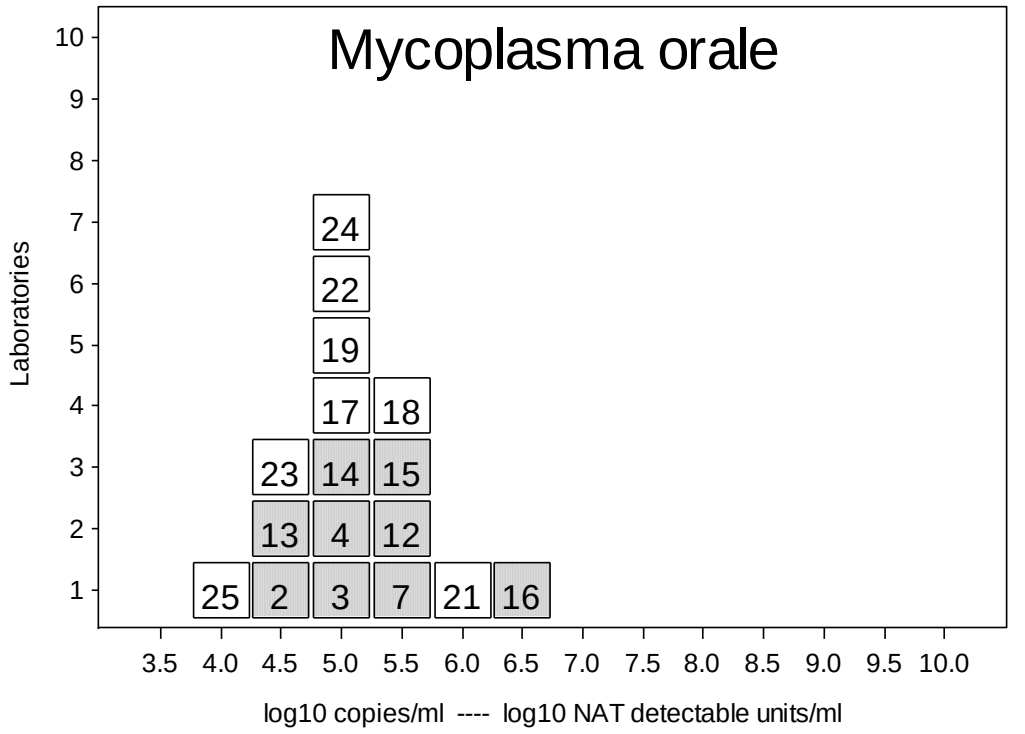
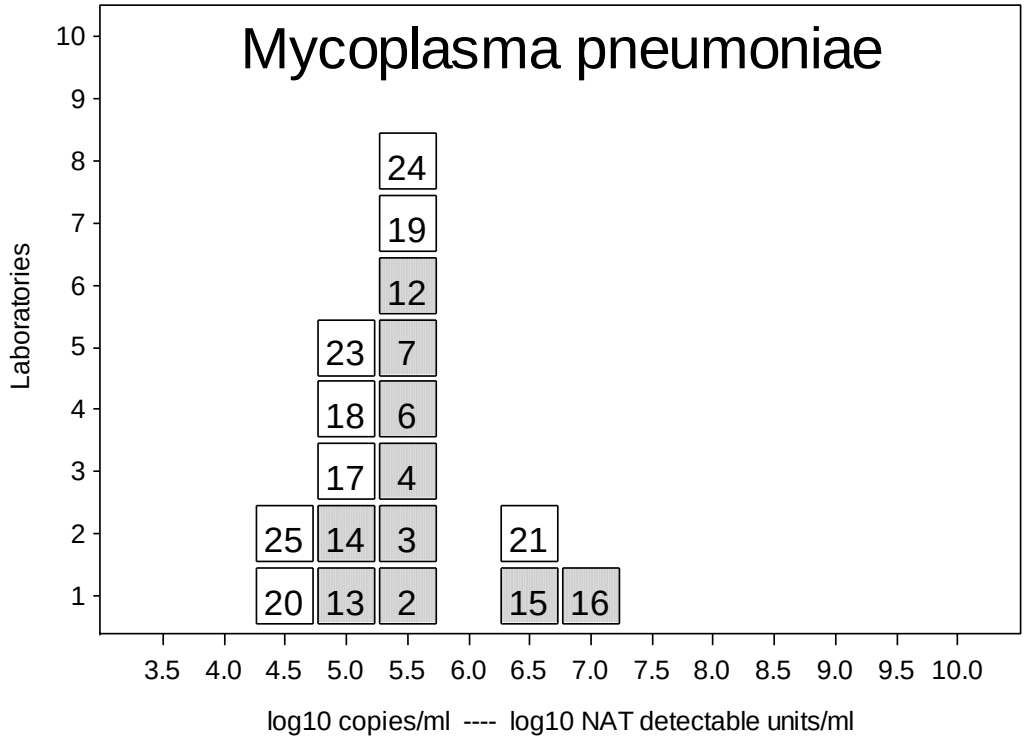

Mycoplasma pneumoniae
Calibrator: M. fermentans
(10^5 Candidate IU / ml)
Laboratories
Relative Potency [log10 Candidate IU/ml]
9
8
5
3
3 4
19 4
18 25
17 23
16 20
16 15
13 15 21
24 11 13 1 2

Figure 8
Combined absolute potency evaluation for quantitative and qualitative NAT assays







Results from quantitative (copies/ml, shaded in grey) and qualitative assays (adjusted for dilution, not shaded).

Attachments

Feasibility study protocols

(a) (Semi)Quantitative NAT assays

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

(Semi)quantitative NAT assays

Objective

To evaluate a panel of lyophilized *Mollicutes* species to determine a suitable species to develop into a World Health Organization (WHO) International Standard (IS). The focus of the study mainly concerns relative amplification and detection efficiency for different *Mollicutes* species by nucleic acid amplification technique (NAT) based assays, rather than limit of detection determination of the individual NAT assay. Pre-analytical enrichment steps like centrifugation of bacteria from a large volume or propagation of bacteria prior to NAT detection are not covered by this study.

Background

Currently there is no standardization of NAT based assays for the detection of *Mollicutes* nucleic acids. A proposal drafted by the Paul-Ehrlich-Institut to develop an IS for NAT assays designed for the detection of *Mollicutes* nucleic acid amplification technique (NAT)-based assays was endorsed by the WHO Expert Committee on Biological Standardization (ECBS) at their meeting in Geneva in October 2010.

In this international collaborative study a panel of *Mollicutes* species will be evaluated by different NAT assays. The purpose of this exercise is to review assay performance and to identify a *Mollicutes* species which has the potential to be a suitable IS.

The panel members represent cultured *Mollicutes* species that have been diluted in *Mollicutes* negative culture media. Therefore, different concentrations of target nucleic acids are present in the panel, together with negative controls. All samples have been given a random panel member number.

Materials

The panel is composed of 9 vials (coded No. 1 to No. 9) each containing lyophilized material representing an original volume of 0.5 ml.

Participants using a (semi-)quantitative NAT assay will receive three panels.

Storage of Materials

The material has been shown to be stable under ambient conditions for several days. After receipt the lyophilized panels should be stored at or below -20 °C until use.

Caution

These preparations contain material which may be infectious for humans. These preparations should be regarded as potentially hazardous to health. They should be used and discarded according to your own laboratory safety procedures. Surface decontamination of the vials has been performed using a disinfectant containing formaldehyde.
These materials are not for *in vitro* diagnostic use, they are for evaluation purposes only and should not be used to determine the validity of NAT assays for *Mollicutes*.

Study protocol

Participants are requested to perform testing of the panel using their routine assay for *Mollicutes* nucleic acids. Each panel should be tested in one run on a different day (resulting in three runs performed on three separate days.)

Reconstitution

On the day of testing, please reconstitute the individual panel members by adding 0.5 ml H_2O (nuclease-free) and keep at room temperature for 10 minutes with gentle shaking, followed by incubation on ice.

Dilution and Testing

Panel members No. 1, 2, 3, 4, 5 should be tested without further dilution.

Panel members No. 6, 7, 8, 9 should be tested both neat and as 1:10 dilution (e.g. 0.1 ml reconstituted panel member material plus 0.9 ml dilution matrix).

For the dilutions please use the usual test matrix of your laboratory, e.g. negative cell culture media.

Please analyse in total these 13 panel samples by using your routine NAT assay.

Replicate extractions and/or replicate amplification/detection may be performed; analysis of any replicates should be indicated in the accompanying results forms.

Some extraction protocols may request larger extraction volumes than provided by the reconstituted panel members.
In this case the extraction protocol may be either adapted to the smaller volume available after reconstitution, or, alternatively, the panel materials may be diluted with negative matrix to obtain larger volumes.
Either modification of the standard protocol should be reported in the Method Reporting Sheet.

If the test results of the first run are above the higher end of the linear range of the assay, for one or more panel members(s), an appropriate dilution (e.g. 1:1,000) dilution, of the respective sample should be chosen for the subsequent runs and indicated in the Result Reporting Sheet.

Results

Results of each assay run should be recorded in a separate Result Reporting Sheet. The results should be reported in a (semi-)quantitative way depending on the assay design, e.g. as a concentration of target nucleic acids (copies/ml, genome equivalents/ml) and/or as C_T -values for real time PCRs. Please report the concentration of the starting material (before extraction).

Data should be returned by 30.07.2012

If any aspect of the protocol is unclear or you have questions, please send an email with your request to M. Nuebling: nuemi@pei.de

All completed forms should be sent (preferably electronically) to: nuemi@pei.de

or by fax: +49 6103 77 1280

or by mail to:

Dr C. Micha Nübling
Paul-Ehrlich-Institut, „Molecular Virology“
Federal Institute for Vaccines and Biomedicines
Paul-Ehrlich-Str. 51-59
63225 Langen, Germany

Thanks a lot in advance for your cooperation !!

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

(Semi)quantitative NAT assays

Method Reporting Sheet

Laboratory: Date:

Operator: Analyst:

Contact person's email address:

Short description of in-house NAT / Test Kit

Extraction platform:

Amplification/detection system:

Region of genome amplified:

Assay reference (if available):

Amplification of □ DNA □ RNA

Volume used for nucleic acid extraction:

Elution volume of nucleic acids:

Volume of eluted nucleic acid used for amplification / (rev. transcr).:

Dilution matrix used:

Current control / calibration material (e.g. extracted *Mollicutes* culture, plasmid DNA, recombinant bacteriophage etc.):

(Please use additional sheets as necessary)

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

(Semi)quantitative NAT assays

Result Reporting Sheet

Run 1 / Panel 1

Name of Participant:
Date of Assay:

	Result (cps/ ml, ct-value, ...)	Result of replicates, if performed; remarks	
Panel Member No. 1			
Panel Member No. 2			
Panel Member No. 3			
Panel Member No. 4			
Panel Member No. 5			
Panel Member No. 6			
Panel Member No. 7			
Panel Member No. 8			
Panel Member No. 9			
Panel Member No. 6, 1:10			
Panel Member No. 7, 1:10			
Panel Member No. 8, 1:10			
Panel Member No. 9, 1:10			

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

(Semi)quantitative NAT assays

Result Reporting Sheet

Run 2 / Panel 2

Name of Participant:
Date of Assay:

	Result (cps/ ml, ct-value, ...)	Result of replicates, if performed; remarks	
Panel Member No. 1			
Panel Member No. 2			
Panel Member No. 3			
Panel Member No. 4			
Panel Member No. 5			
Panel Member No. 6			
Panel Member No. 7			
Panel Member No. 8			
Panel Member No. 9			
Panel Member No. 6, 1:10			
Panel Member No. 7, 1:10			
Panel Member No. 8, 1:10			
Panel Member No. 9, 1:10			

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

(Semi)quantitative NAT assays

Result Reporting Sheet

Run 3 / Panel 3

Name of Participant:
Date of Assay:

	Result (cps/ ml, ct-value, ...)	Result of replicates, if performed; remarks	
Panel Member No. 1			
Panel Member No. 2			
Panel Member No. 3			
Panel Member No. 4			
Panel Member No. 5			
Panel Member No. 6			
Panel Member No. 7			
Panel Member No. 8			
Panel Member No. 9			
Panel Member No. 6, 1:10			
Panel Member No. 7, 1:10			
Panel Member No. 8, 1:10			
Panel Member No. 9, 1:10			

Attachments

Feasibility study protocols

(b) Qualitative NAT assays

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

Qualitative NAT assays

Objective

To evaluate a panel of lyophilized *Mollicutes* species to determine a suitable species to develop into a World Health Organization (WHO) International Standard (IS). The focus of the study concerns relative amplification and detection efficiency for different *Mollicutes* species by nucleic acid amplification technique (NAT)-based assays. Pre-analytical enrichment steps like centrifugation of bacteria from a large volume or propagation of bacteria prior to NAT detection are not covered by this study.

Background

Currently there is no standardization of NAT based assays for the detection of *Mollicutes* nucleic acids. A proposal drafted by the Paul-Ehrlich-Institut to develop an IS for NAT assays designed for the detection of *Mollicutes* nucleic acid amplification technique (NAT)-based assays was endorsed by the WHO Expert Committee on Biological Standardization (ECBS) at their meeting in Geneva in October 2010.

In this international collaborative study a panel of *Mollicutes* species will be evaluated by different NAT assays. The purpose of this exercise is to review assay performance and to identify a *Mollicutes* species which has the potential to be a suitable IS.

The panel members represent cultured *Mollicutes* species that have been diluted in *Mollicutes* negative culture media. Different concentrations of target nucleic acids may be present in the panel, together with negative controls. All samples have been given a random panel member number.

Materials

The panel is composed of 5 vials (coded No. 1, 2, 3, 4 and 9); each containing lyophilized material representing an original volume of 0.5 ml.

Participants using a qualitative NAT assay will receive three panels.

Storage of Materials

The material has been shown to be stable under ambient conditions for several days. After receipt, the lyophilized panels should be stored at or below -20 ºC until use.

Caution

These preparations contain material which may be infectious for humans. These preparations should be regarded as potentially hazardous to health. They should be used and discarded according to your own laboratory safety procedures. Surface decontamination of the vials has been performed using a disinfectant containing formaldehyde.

These materials are not for *in vitro* diagnostic use, they are for evaluation purposes only and should not be used to determine the validity of NAT assays for *Mollicutes*.

Study protocol

Participants are requested to perform testing of the panel using their routine assay for *Mollicutes* nucleic acids. Each panel should be tested in one run on a different day (resulting in three runs performed on three separate days). The design of the 2nd and 3rd run depends on the results obtained in the previous run.

Reconstitution

On the day of testing, please reconstitute the individual panel members by adding 0.5 ml H_2O (nuclease-free) and keep at room temperature for 10 minutes with gentle shaking, followed by incubation on ice.

First run

Dilution and testing

For the first run, please perform 1 $\log_{10}$ dilution (1:10) series of the panel members as follows:

Panel members 1, 2, 3, 4: from 10^{-1} to 10^{-4}

Panel member 9: from 10^{-1} to 10^{-6}.

For the dilution please use the usual test matrix of your laboratory, e.g. negative cell culture media.

For example, dilution series may be performed by pipetting 0.1 ml reconstituted neat material into 0.9 ml dilution matrix resulting in the 10^{-1} dilution, followed by analogous subsequent dilution step(s).

In the first run of extraction and amplification, please **analyse** the following panel samples:

Panel members 1, 2, 3, 4: neat, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilution

Panel member 9: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}.

Replicate extractions and/or replicate amplification/detection may be performed; analysis of any replicates should be indicated in the accompanying results forms.

Some extraction protocols may request larger extraction volumes than provided by the reconstituted panel members.
In this case, the extraction protocol may be either adapted to the smaller volume available after reconstitution, or, alternatively, the panel materials may be diluted with negative matrix to obtain larger volumes.
Either modification of the standard protocol should be reported in the Method Reporting Sheet.

In the first run, please determine the preliminary end point dilution (lowest concentration with a positive NAT result) for the different panel members.
This end point dilution will be used for calculation of the panel member specific dilutions for the 2nd run performed with another panel (see instructions below).

Notes for determination of the end point dilution in Run 1

If (a) panel member is consistently negative, please use the same $\log_{10}$ dilutions as in the first run for the 2nd run (diluting members of the 2nd panel).
If all proposed dilutions of a panel member are tested consistently positive, please use 3 additional $\log_{10}$ dilutions of this panel member for the 2nd run and do not analyse again the three highest concentrations of the analyte from the first run.
If a panel member yields discontinuous results (a negative result followed again by a positive result for the next higher dilution), then for the end-point determination please ignore this positive result (possibly due to Poisson distribution of the analyte); however, please indicate this result in the Results Reporting Sheet.
If replicate testing is performed, generating discrepant test results for the lowest positive concentration, this will be defined as the end point dilution.

Second run

Dilution and Testing

Please test for each Panel Member the specific end point dilution (determined in Run 1) together with two half-log_{10} dilutions around the end point dilution.

This approach may be explained by an example for a Panel Member with a hypothetical end point dilution of 10^{-2}, as determined in Run 1

Log dilutions of 1st run	**Results of 1st run**	**Half-log_{10} dilutions to be tested in the 2nd run using 2nd panel**
neat	**pos**	
10^{-1}	**pos**	10^{-1}
		$10^{-1,5}$
10^{-2}	**pos**	**10^{-2}**
		$10^{-2.5}$
10^{-3}	neg	10^{-3}
10^{-4}	neg	

In this example **five half-log_{10} dilutions** (1:3.16) would be prepared (1 volume analyte + 2.16 volume dilution matrix) ranging from 10^{-1} to 10^{-3}, e.g. by diluting 200 µl of the 10^{-1} dilution using 432 µl dilution matrix, and subsequently repeated for the following lower concentrations.

The starting concentration (in this example 10^{-1} dilution of respective panel member) may be prepared by 1 log_{10} dilution(s).

At least five concentration levels differing by half-log_{10} will be analysed.

Additional levels may be tested; in this case please report these additional results as well.

The Notes of the first run apply as far as appropriate.

Third run

Dilution and Testing

Please test for each Panel Member the specific end point dilution (as determined in Run 2) together with 2 half-log_{10} dilutions around the end point dilution. The end point dilution determined in Run 2 may differ from Run 1.

Results

Results of each assay run should be recorded in a separate Result Reporting Sheet. For qualitative NAT assays the results should be reported positive, negative or equivocal. If the assay design allows further conclusions (e.g. differentiation between strong or weak positive results) please report this information in the result sheet, too.

Data should be returned by 30.07.2012

If any aspect of the protocol is unclear or you have questions, please send an email with your request to M. Nuebling: nuemi@pei.de

All completed forms should be sent (preferably electronically) to: nuemi@pei.de

or by fax: +49 6103 77 1280

or by mail to:
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Federal Institute for Vaccines and Biomedicines
Paul-Ehrlich-Str. 51-59
63225 Langen, Germany

Thanks a lot in advance for your cooperation !!

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

Qualitative NAT assays

Method Reporting Sheet

Laboratory: Date:

Operator: Analyst:

Contact person's email address:

Short description of in-house NAT / Test Kit

Extraction platform:

Amplification/detection system:

Region of genome amplified:

Assay reference (if available):

Amplification of □ DNA □ RNA

Volume used for nucleic acid extraction:

Elution volume of nucleic acids:

Volume of eluted nucleic acid used for amplification / (rev. transcr).:

Dilution matrix used:

Current control / calibration material (e.g. extracted *Mollicutes* culture, plasmid DNA, recombinant bacteriophage etc.):

(Please use additional sheets as necessary)

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

Qualitative NAT assays

Run 1 / Panel 1

Name of Participant:
Date of Assay:

Dilution	Result Panel Member No. 1	Result Panel Member No. 2	Result Panel Member No. 3	Result Panel Member No. 4	Result Panel Member No. 9
neat					
10^{-1}					
10^{-2}					
10^{-3}					
10^{-4}					
10^{-5}					
10^{-6}					

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

Qualitative NAT assays

Run 2 / Panel 2

Name of Participant:
Date of Assay:

Dilution	Result Panel Member No. 1	Result Panel Member No. 2	Result Panel Member No. 3	Result Panel Member No. 4	Result Panel Member No. 9
neat					
$10^{-0.5}$					
10^{-1}					
$10^{-1.5}$					
10^{-2}					
$10^{-2.5}$					
10^{-3}					
$10^{-3.5}$					
10^{-4}					
$10^{-4.5}$					
10^{-5}					
$10^{-5.5}$					
10^{-6}					
$10^{-6.5}$					

WHO Feasibility Study to Evaluate a *Mollicutes* Species Panel

Qualitative NAT assays

Run 3 / Panel 3

Name of Participant:
Date of Assay:

Dilution	Result Panel Member No. 1	Result Panel Member No. 2	Result Panel Member No. 3	Result Panel Member No. 4	Result Panel Member No. 9
neat					
$10^{-0.5}$					
10^{-1}					
$10^{-1.5}$					
10^{-2}					
$10^{-2.5}$					
10^{-3}					
$10^{-3.5}$					
10^{-4}					
$10^{-4.5}$					
10^{-5}					
$10^{-5.5}$					
10^{-6}					
$10^{-6.5}$					

Instruction for Use (draft)

Paul-Ehrlich-Institut
Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

A WHO Collaborating Centre
for Quality Assurance of Blood Products and
in vitro Diagnostic Devices

1st World Health Organization International Standard for mycoplasma DNA for nucleic acid amplification technique (NAT)-based assays
PEI code 8293/13
Version 1.0, 06th August 2013

1. INTENDED USE

The 1st World Health Organization (WHO) International Standard (IS) for Mycoplasma DNA (8293/13) is intended to be used in the standardization of nucleic acid amplification technique (NAT)-based assays of generic design, allowing the simultaneous detection of distantly related mycoplasma species. This WHO IS was generated from *Mycoplasma fermentans* (strain PG18^T, NCTC 10117). The need to develop a standard was demonstrated in previous studies (1) and in the feasibility study of this standardization project (2).
Mycoplasma fermentans is a bacterial species which may infect humans (without clear pathogenic potential) and which has been described as one of the more frequent mycoplasma contaminants of eukaryotic cell cultures. Mycoplasma contamination of cell cultures may alter cellular parameters, and therefore mycoplasma-free cell cultures are required both in research and in manufacturing environments. In different regions of the worl, regulations are in place to assure mycoplasma-free cell cultures used for the manufacture of biomedicinal products. In the more recent past, culture-based approaches of mycoplasma testing have been supplemented or in some cases even replaced by NAT tests designed for generic detection of mycoplasma species (3).
The project for the 1st World Health Organization (WHO) International Standard (IS) for mycoplasma DNA for nucleic acid amplification technique (NAT)-based assays was endorsed by the Expert Committee on Biological Standardization of the WHO in October 2010. The collaborative study performed for the establishment of the WHO IS consisted of two parts: a feasibility study was followed by a comparability study. In the feasibility study NAT assays of different design and of worldwide representation were evaluated with different mycoplasma species. In this study harmonisation of assays (reduction of interassay variation) was demonstrated and the candidate species for the WHO IS was selected. The comparability study confirmed the suitability of the candidate preparation and enabled its assignment of International Units. Further details of the collaborative study are available in the report WHO/BS/YY.XXXX (2).

2. UNITAGE

This reagent has been assigned a unitage of 200,000 International Units/ml (2 x 10^5 IU/ml).

3. CONTENTS

Each vial contains 0.5 ml of lyophilized *Mycoplasma fermentans* in Mycosafe Friis culture medium. The bacteria were harvested during the exponential growth phase.

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.
The preparation contains lyophilized bacteria which may be potentially infectious and pathogenic for humans. The reference material has been grown in Mycosafe Friis medium tested negative for mycoplasma DNA prior to its use.
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 probably will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vx
ials, to avoid cuts.

5. 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 should be stored at or below -20ºC.

The WHO IS should be reconstituted with 0.5 ml of sterile nuclease-free water.

If all the material is not used immediately, laboratories may aliquot the remaining material into suitable volumes which should be stored at or below -70ºC.
In the collaborative study (2) the WHO IS candidate was characterized with using the routine test matrixes of the laboratories as diluents. These diluents included isotonic buffers, saline, culture medium, cultured cells, cell culture supernatant or virus bulk harvest, without detrimental effects on mycoplasma NAT detection observed.

6. STABILITY

As the stability studies with accelerated conditions indicate high stability of the lyophilized reference material under the recommended storage conditions (at or below-20ºC), there is no expiry date assigned to the International Standard. This approach complies with the recommendations for the preparation, characterization and establishment of international and other biological reference standards (4). The International Standard is held at the Paul-Ehrlich-Institut (PEI) within assured, temperature-controlled storage facilities. During its life cycle the stability is monitored at regular intervals. The International Standard remains valid with the assigned potency and status until withdrawn or amended.

Reference materials should be stored on receipt as indicated on the label. Once, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact PEI.

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Federal Institute for Vaccines and Biomedicines

A WHO Collaborating Centre
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in vitro Diagnostic Devices

7. REFERENCES

(1) Milne C, Daas A. Establishment of European Pharmacopeia Mycoplasma Reference Strains. Pharmeuropa Bio 2006, 1:57-71.
(2) Hanschmann KM, Montag-Lessing T, Baylis SA, Chudy M, Kreß J, Ulrych U, Czurda S, Rosengarten R, Nübling CM. (2013) Collaborative Study to Establish the 1st World Health Organization International Standard for Mycoplasma DNA for Nucleic Acid Amplification Technique (NAT)-Based Assays. WHO Report 2013, WHO/BS/YY.XXXX
(3) Volokhov D, Graham LJ, Brorson KA, Chizhikov VE. Mycoplasma testing of cell substrates and biologics: Review of alternative non-microbiological techniques. Molecular and Cellular Probes 2011, 25:69-77.
(4) Recommendations for the preparation, characterization and establishment of international and other biological reference standards. WHO Expert Committee on Biological Standardization. Fifty-fifth report, 2004. (WHO Technical Report Series, No. 932).

8. FURTHER INFORMATION

About this material: whoccivd@pei.de
About WHO Biological Reference Preparations: http://www.who.int/biologicals/en/

9. 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 whoccivd@pei.de

10. CITATION

In any circumstance where the recipient publishes a reference to any WHO IS provided by the WHO Collaborating Centre PEI, it is important that the title of the preparation and the PEI code number, and the name and address of PEI are cited correctly.

11. MATERIAL SAFETY SHEET

Physical properties (at room temperature)		
Physical appearance	Lyophilized powder	
Fire hazard	None	
Chemical properties		
Stable	Yes	Corrosive:No
Hygroscopic	No	Oxidising:No
Flammable	No	Irritant: No
Other (specify) CONTAINS MYCOSAFE FRIIS MEDIUM AND INFECTIOUS MYCOPLASMA FERMENTANS		
Handling: See caution, section 4		
Toxicological properties		
Effects of inhalation: Avoid – *contains infectious Mycoplasma fermentans*		
Effects of ingestion: Avoid – *contains infectious Mycoplasma fermentans*		
Effects of skin absorption: Avoid – *contains infectious Mycoplasma fermentans*		

Suggested First Aid
Inhalation Seek medical advice - *contains infectious Mycoplasma fermentans*
Ingestion Seek medical advice - *contains infectious Mycoplasma fermentans*
Contact with eyes Wash thoroughly with water; seek medical advice – *contains infectious Mycoplasma fermentans*
Contact with skin Wash thoroughly with water; seek medical advice – *contains infectious Mycoplasma fermentans*
Action on Spillage and Method of Disposal
Spillage of vial 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.

12. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependent on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of the Institute in connection with this agreement, whether for negligence or breach of agreement or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

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