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Rapid Mycoplasma Testing Service Delivers Speed, Sensitivity and Specificity to a Broad Scope of Sample Matrices

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Mycoplasmas are the smallest known self-replicating prokaryotes. They parasitize a wide range of host organisms, including humans, animals, plants and insects and are common contaminants of mammalian cell culture. Due to their small size, mycoplasmas can readily pass through 0.2 µm filters used to maintain sterility. Sources for the introduction of mycoplasmas into cell culture systems include: cell culture media and additives, the use of previously infected cells, and laboratory personnel.

Although visual signs indicative of mycoplasma contamination are often lacking, mycoplasma contamination of cell lines used to produce biopharmaceutical products can disrupt cellular growth and metabolism and lead to changes in gene expression. These adverse cytopathological events can result in decreased product quantity and quality.

More importantly, mycoplasma infections have been associated with respiratory illness, urethritis, and arthritis, and they act as a cofactor in numerous infectious diseases. It is for these reasons that world-wide regulatory agencies require that biotechnological products produced in cell substrates be tested to ensure the absence of mycoplasma contamination.

Compendial mycoplasma testing procedures for the detection of viable mycoplasma contaminants are culture-based and have three components: direct cultivation on agar plates, enrichment in broth followed by detection on agar plates, and detection in indicator cell culture.

The current procedures are time consuming as they are 28 days in length. This time requirement is not amenable for obtaining the rapid lot release testing results needed for biopharmaceutical products that have short half-lives or for which there is high market demand.

The lengthy assay period is also not conducive to the rapid screening of raw materials intended for use in future production, nor to the rapid in-process screening of intermediates for the purpose of detecting and containing contamination events. Eurofins BioPharma Product Testing offers a 5-day rapid mycoplasma test comparable in sensitivity and specificity to the 28-day compendial method.

The MycoSEQ™ kit is able to detect *Mycoplasma* species in a simple, reliable, and rapid manner and can test variable volumes, from 100 mL to 10 mL of cell culture containing 10^8 cells. The kit utilizes a multiparameter approach for determination of positive and negative species. The kit also includes a Discriminatory Positive/Extraction Control (DPC). This DPC is a large plasmid with a mycoplasma DNA sequence in order to behave like mycoplasma DNA through extraction and detection. This sequence has been modified to ensure that the melting temperature (T_m) is outside the range of the expected amplicon T_m values from mycoplasma species.

Extraction of nucleic acids uses the PrepSEQ™ kit and the proprietary magnetic bead-based separation technology. The kit is scalable from 100µL to volumes over 10 mL. A differential lysis protocol captures DNA from both cell-associated and free Mycoplasma for an efficient extraction from the test sample. Extraction process is semi-automated to minimize handling and possible cross-contamination. Samples and/or

controls can be spiked with the DPC to ensure that the extraction protocol is performing as intended. The DPC spike is a risk free DNA spike that eliminates the possibility of false positives from cross-contamination with typical positive control DNA.

To optimize the number of mycoplasma species detected, an exhaustive bioinformatics analysis was undertaken for designing the primers and reaction conditions. This analysis allows for detection of over 90 species, as well as the related *Acholeplasma laidlawii* and *Spiroplasma citri*. The detection limit for the kit is at least 1-10 genome copies per reaction. Sensitivity will vary depending upon the source sample, but the sample preparation can allow for detection of the species at 10 CFU/mL (which is keeping with the compendial mycoplasma detection method limits).

The AccuSEQ™ software provides a presence/absence results on samples post qPCR run. The algorithms designed for this software were created from data generated with the MycoSEQ™ Mycoplasma detection assay. In this assay, the determination of presence or absence of mycoplasma is based upon a triad of information. The T_m and derivative value (DV) for each sample (which are derived through the use of SYBR Green) allow for differentiation of true signal from background noise or contamination with the DPC. While the C_t value for test samples (when present) or inhibition controls can determine if/when a sample signal rises above a background fluorescence level. These three pillars of information provide the basis for the AccuSEQ software to determine if a sample/control contains Mycoplasma DNA, DPC DNA, or absent of either DNA. An easy manual review process is available that contains all T_m and C_t values, as well as the amplification, multicomponent, and raw data plots.

Eurofins BioPharma Product Testing has utilized its experience in Mycoplasma testing to develop a protocol for testing neat and a diluted matrix in order to test for interference in readings due to inhibitory effects from sample matrix. This interference study utilizes the sample and dilution spiked with the DPC prior to extraction. The DPC spiked sample must generate a positive result within a limit related to the positive control in order for the sample to have no matrix interference.

Applied Biosystems/ThermoFisher externally validated the assay following guidance provided in E.P. 2.6.7. This validation verified the level of detection (LOD) for both genome copies and live mycoplasma stocks in a matrix of CHO cells. This validation demonstrated a comparison to the official culture method of at least 10 CFU/mL total cells detection for the 10 Mycoplasma tested species. This validation also demonstrated specificity when used in three mammalian cells lines, seven bacterial species, and one yeast species.

Within Eurofins BioPharma Product Testing, the method has been validated as well for a qualitative limit of detection (≤ 10 CFU/mL) for *M. pneumonia, M. orale, M. hyorhinis α, and S. melliferum*. Specificity was tested in C. sporopgenes (passed). And robustness was tested with this assay. The extractions were placed at -20°C for one week. The frozen samples and freshly tested samples had results that were similar.

The MycoSEQ kit is a comparable method for a rapid, robust, and reliable mycoplasma detection system. The kit meets E.P. 2.6.7 guidance and achieves the sensitivity of the compendial testing at a fraction of the time, while retaining the compendial volumes for testing. The triad of information (C_t, T_m, DV) provides a confidence in the sensitivity for the detection of fewer than 10 genome copies per reaction.