VALIDATION OF MYCOPLASMA DETECTION KIT IN ACCORDANCE WITH ICH Q2 (R1) METHODOLOGY & ANALYTICAL PROCEDURES GUIDELINES

This validation protocol has been adopted in line with the Methodology and Analytical Procedures Guideline Q2(R1) developed by ICH Expert Working Group.

Document History

First Codification	History	Date
Version#1 **Version#2** **Version#3**	**VALIDATION DATA OF KRISHGEN MYCOPLASMA DETECTION KIT (Cat No#CC0010/CC0020/CC0030)** **Approved by QC and renamed as per ICH guidelines Q2(R1)** **Approved by QC and renamed as per ICH guidelines Q2(R1)**	16th Sept, 2012 27th Dec, 2012 14th Jan, 2013

Approved Quality Control	Approved Product Development	Approved Operations Head
		
14.01.2013	14.01.2013	14.01.2013

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Introduction :

The KRISHGEN Mycoplasma Detection Kit is for detection of mycoplasma contamination in biological samples. The test procedure was developed by us.

This validation guide summarizes the results of our kit in line with European Pharmacopeia 2.6.21 guidelines for detection assays and in accordance with ICH Q2(R1) guidelines.

The document is prepared based on tests run in our laboratory and does not necessarily seek to cover the testing that may be required at user's end for registration in, or regulatory submissions. The objective of this validation is to demonstrate that it is suitable for its intended purpose – determination of mycoplasma in cell culture.

Validation characteristics considered by us in accordance with the guidelines are listed below :

- **Specificity**
- **Sensitivity**
- **Precision**

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

For any queries or support on the data and its performance, please contact us at sales@krishgen.com

Background :

Mycoplasmas are parasitic bacteria that frequently contaminate tissue cultures and consequently also virus stocks, vaccines, and other biological materials produced in cells. The contamination can originate from serum, laboratory staff, other contaminated cultures, or the donors fomr which the cells have been harvested, and it often remains unnoticed.

Although mycoplasmas do not cause visible cell damage, they seriously affect cell growth and metabolism, transfection efficiency, protein and monoclonal antibody synthesis , cytokine secretion, immunological properties, signal transduction, virus proliferation and can even cause DNA / RNA damage.

Since mycoplasmas are very small and lack cell walls, detection using a conventional light microscope is nearly impossible. In addition, cloudiness in the medium, typical for bacterial or yeast contaminations, does not occur even at high Mycoplasma concentrations.

PCR on the other hand is a highly sensitive, specific, and rapid method for the detection of Mycoplasma contamination in culture supernatant, cell/virus stocks, and medium. All cell / tissue

culture relevant Mycoplasma species can be detected in the assay including M. fermentans, M. hyorhinis, M. arginini, M. orale, M. salivarium, M. hominis, M. pulmonis, and M. pirum. In addition, M pneumoniae, acholeplasma laidlawii, M. synoviae, and Ureaplasma species can be detected.

Principle of the Detection Procedure Used :

KRISHGEN Mycoplasma Detection Kit employs a nucleic acid amplification test on the basis of a polymerase chain reaction. We utilize conventional PCR technology for convenient and specific detection of more than 25 containing Mycoplasma species.

The primer set is specific for the highly conserved 16S-rRNA coding region in the Mycoplasma genome.

An Internal Control indicated by a distinct 479bp band derived from the IC DNA on the agarose gel is run with every PCR reaction to confirm a successful PCR reaction.

Sample Preparation :
Cell lines were pre-cultured in the absence of Mycoplasma active antibiotics for 2 passages to maximize test sensitivity.

For later PCR analysis stable templates were prepared by boiling the supernatant of cell cultures / other biological for 10 minutes as per procedure internalized.

Note: Samples were derived from cultures that are at 90 – 100 % confluence. PCR inhibiting substances may accumulate in the medium of older cultures. For samples from such cultures or samples containing high amounts of serum (eg > 205 FBS), proteins or DMSO, a DNA extraction was performed prior to testing.

Operational Details used for the Validation :

Validation Guide / Document No	: KBMB1301
Guide / Document Release Dated	: 16th Sept 2012 / 14th Jan 2013
Kit Validated	: Mycoplasma Detection in Cell Culture Sample using PCR
Samples Used (*species)*	: DNA extracted from the Mycoplasma (Refer Table 1)
Instrument Used	: Biorad Thermal Cycler MWG Biotech Thermal Cycler Biorad Gel Electrophoresis System Biorad Gel Doc System Eppendorf Pipettes Norgen Biotek Genomic DNA Isolation Kit

1. **1.1 Specificity of the Assay**
   The PCR assay can detect the DNA from approximately 15 mycoplasma per sample volume. The primer set amplifies a DNA sequence within the highly conserved 16S- rRNA operon coding region in the Mycoplasma genome. Refer

Table 1 for the mycoplasma species detection spectrum. The primers used for this assay were also tested against most common pathogens like E. coli, Salmonella, and Streptococcus agalactiae; genomic DNA of cell lines like CHO; genomic DNA from human and found no cross reactivity with any of the species tested.

Table 1: Different Mycoplasma species used for validation.

Particulars	Specificity	Particulars	Specificity
Mycoplasma orale	+++	Mycoplasma fermentans	++
Mycoplasma fermentans	+++	Mycoplasma gallinaceum	++
Mycoplasma hyorhinis	+++	Mycoplasma gallisepticum	++
Mycoplasma argininii	+++	Mycoplasma gateae	++
Mycoplasma salivarium	+++	Mycoplasma genitalium	++
Mycoplasma hominis	+++	Mycoplasma glycophilum	++
Acholeplasma laidlawii	+++	Mycoplasma hyosynoviae	++
Mycoplasma arthritidis	++	Mycoplasma hominis	++
Mycoplasma hominis	++	Mycoplasma hyorhinis	++
Mycoplasma pirum	++	Mycoplasma hyosynoviae	++
Mycoplasma salivarium	++	Mycoplasma insons	++
Acholeplasma axanthum	+	Mycoplasma mobile	++
Acholeplasma granularum	+	Mycoplasma muris	++
Acholeplasma oculi	+	Mycoplasma neurolyticum	++
Mesoplasma coleopterae	++	Mycoplasma opalescens	++
Mycoplasma anatis	++	Mycoplasma ovipneumoniae	++
Mycoplasma arthritidis	++	Mycoplasma orale	++
Mycoplasma bovigenitalium	++	Mycoplasma penetrans	++
Mycoplasma bovirhinis	++	Mycoplasma preputii	++
Mycoplasma bovoculi	++	Mycoplasma primatum	++
Mycoplasma buccale	++	Mycoplasma pirum	++
Mycoplasma californicum	++	Mycoplasma pulmonis	++
Mycoplasma canadense	++	Mycoplasma salivarium	++
Mycoplasma canis	+	Mycoplasma spermatophilum	++
Mycoplasma capricolum	++	Mycoplasma synoviae	++
Mycoplasma caviae	++	Mycoplasma tullyi	++
Mycoplasma columbinasale	++	Spiroplasma cantharicola	+
Mycoplasma columbinum	++	Spiroplasma citri	+
Mycoplasma cloacale	++	Spiroplasma floricola	+
Mycoplasma columborale	++	Spiroplasma ixodetis	+
Mycoplasma cricetuli	++	Spiroplasma lineolae	+
Mycoplasma cynos	++	Spiroplasma monobiae	+
Mycoplasma equirhinis	++	Spiroplasma platyhelix	+
Mycoplasma falconis	++	Spiroplasma tabanidicola	+
Mycoplasma faucium	++	Spiroplasma taiwanense	+
Mycoplasma felis	++	Ureaplasma parvum	+

+++ *denotes high specificity*
++ *denotes moderate specificity*
+ *denotes specificity*

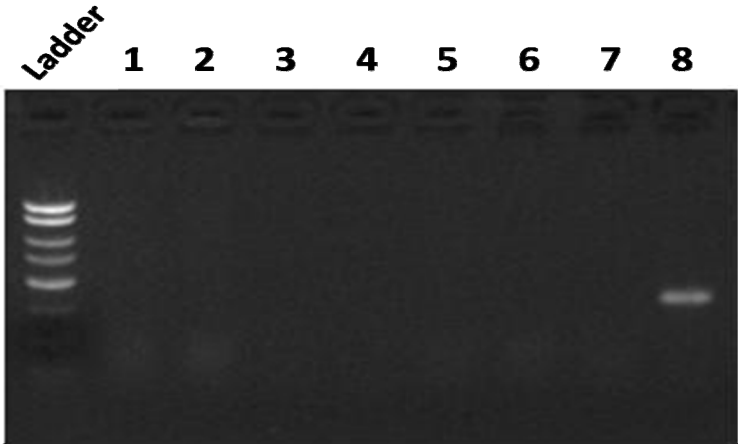

Fig.1: Specificity of the Krishgen Mycoplasma Detection Assay
Lane 1: *Negative sample control;*
Lane 2: *Negative PCR control;*
Lane 3: *Genomic DNA of E.coli;*
Lane 4: *Genomic DNA of Salmonella;*
Lane 5: *Genomic DNA of Streptococcus agalactiae;*
Lane 6: *Genomic DNA of CHO cell line;*
Lane 7: *Genomic DNA of Human;*
Lane 8: *Genomic DNA of Mycoplasma.*

Cross Reactivity :
The kit used was also tested with samples of common pathogens like E.coli, salmonella and streptococcus agalactiae, genomic DNA of cell line of CHO, genomic DNA from human and no cross reactivity was observed with any of the species tested.

1.2 Sensitivity of the Assay

The kit used for the assay has a detection limit of approx. 15fg of Mycoplasma DNA which corresponds to 15 mycoplasma per sample volume.

2. Comparison of the performance parameters to other commercially available PCR technologies

About 20 different samples from different cell cultures were examined. These samples were tested using Krishgen Mycoplasma Detection Kit and another commercial available kit. All the positive results were comparable between the two assays. However, there were 3 false negative samples which were picked up by Krishgen Mycoplasma Detection Kit as it included a sensitive internal control.

Particulars	Krishgen Assay	Assay Performed Using Competitor Assay PCR Kit
Total No Of Samples	20	20
Negative Results	5 (25%)	8 (40%)
Negative Agreement *(Krishgen/Competitor)*	5 (100%)	
Positive Results	15 (75%)	12 (60%)
Positive Agreement *(Krishgen/Competitor)*	15 (100%)	

With the exception of 3 samples, the data for samples tested co-related between the two kits

3. Precision :

As a precision control for the test samples, known mycoplasma positive samples and known negative samples were tested and found satisfactory results using the Krishgen Mycoplasma Detection Kit.

Particulars	Total No Of Positive Samples	Krishgen Results	Positive Agreement (%)
Run No#1	12	12	100%
Run No#2	10	10	100%
Run No#3	15	15	100%

In order to test the accuracy of the results, inter personal validation were also carried out by more than one person in the laboratory.

Particulars	Total No Of Samples	Technician `A'		Technician `B'	
		Positive	Negative	Positive	Negative
Run No#1	5	2	3	2	3
Run No#2	5	2	3	2	3
Run No#3	5	2	3	2	3

Conclusion :

The KRISHGEN Mycoplasma Assay Kit uses a PCR kit and methodology

for the detection of Mycoplasma contamination in biological samples. The performance parameters of this kit was determined in this study.

The PCR technology used by us in this assay kit is characterized by particularly high sensitivity. The detection limit of the kit was studied and defined. The internal control of the kit used by us ensured safe exclusion of false negative results. The kit used in the test is highly reproducible with both handling personnel and individually.

The KRISHGEN Mycoplasma Detection Kit uses a PCR kit which fulfils the prerequisites of nucleic acid amplification techniques.

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Dated : 14th Jan 2013.

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