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Manufacturing

In-house abbreviated qualification of a real-time polymerase chain reaction method and strategies to amplify mycoplasma detection in human mesenchymal stromal cells

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ABSTRACT

Background aims: In this study, the authors performed an in-house abbreviated qualification of a commercially available real-time polymerase chain reaction (PCR) kit for limit of detection (LOD), matrix interference and ruggedness of mycoplasma detection in a human bone marrow-derived mesenchymal stromal cell (MSC (M)) investigational cell product (NCT02351011). The approach used was similar to an abbreviated qualification the authors previously conducted for endpoint PCR, which was accepted by Canadian regulators for final product release of the same MSC(M) investigational cell product for treatment of osteoarthritis patients (NCT02351011). With patient consent, biobanked MSCs(M) were re-analyzed by real-time PCR for mycoplasma detection to conduct in-house qualification of the kit.

Methods: LOD was determined by spiking MSCs(M) with a series of 10-fold dilutions of two commercially available genomic DNA (gDNA) reference standards for *Mycoplasma arginini* (*M. arginini*) and *Mycoplasma hominis* (*M. hominis*). Matrix interference was tested by using 10-fold dilutions of MSC(M)s down to 4500 cells/mL. Polyadenylic acid (poly[A]) was used to improve DNA recovery in samples with 4500–45 000 MSCs(M)/mL. Real-time PCR tests performed on different days were compared to evaluate ruggedness.

Results: Real-time PCR analysis showed a conservative LOD of 40 genome copies (GCs)/mL and 240 GCs/mL, which are equivalent to 10 colony-forming units (CFUs)/mL, for *M. arginini* and *M. hominis*, respectively. According to a less conservative manufacturer-based criterion for positivity, the kit detected 0.4 GC/mL (0.1 CFU/mL) and 24 GCs/mL (1 CFU/mL) *M. arginini* and *M. hominis*, respectively. Real-time PCR with different MSC(M) dilutions did not show matrix interference. However, DNA recovery was compromised at MSC(M) concentrations at or below 45 000 cells/mL. The addition of poly(A) as a DNA carrier improved DNA recovery and allowed an LOD, considered here to be equivalent to 10 CFUs/mL, to be achieved, which was not possible in diluted MSC(M) samples ($\leq 45\,000$ cells/mL) in the absence of poly(A). Ruggedness was demonstrated with tests ($n = 18$) performed on different days, with an average overall inter-assay percent coefficient of variation of less than 4 for *M. arginini* (3.62 [400 GCs/mL], 3.61 [40 GCs/mL]) and less than 3 for *M. hominis* (2.83 [2400 GCs/mL], 1.95 [240 GCs/mL]).

Conclusions: A commercially available real-time PCR mycoplasma detection kit was qualified for evaluating mycoplasma contamination in investigational MSC(M) products and met the criteria used previously (and accepted by Canadian regulators) for in-house qualification of an endpoint PCR mycoplasma detection kit, and the addition of poly(A) addressed the poor recovery of mycoplasma gDNA in samples with low cell numbers.

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Introduction

Culture-expanded human mesenchymal stromal cells (MSCs) are being evaluated as advanced therapy medicinal products for multiple indications [1]. As part of clinical evaluation and subsequent clinical and commercial translation, MSC products must demonstrate

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mycoplasma-free status using validated tests to meet regulatory requirements harmonized across multiple jurisdictions [2–5]. Mycoplasma contamination in cell cultures remains a concern with increasing numbers of approved cell therapy clinical trials [6]. Agar culture and indicator cell culture methods are the gold standard tests for detecting cultivable and non-cultivable mycoplasmas, respectively [7]. These tests are typically time-consuming and can cause delays in product release, especially with regard to just-in-time manufactured autologous cell therapy products [8].

Nucleic acid amplification techniques (NATs) are accepted by regulators as an alternative to the culture and indicator methods for detection of mycoplasmas [9]. NAT methods come under the category of rapid microbiological methods, which can provide equivalent or better results than compendial methods in less time [10]. The authors previously conducted an in-house, abbreviated qualification of a commercially available and fully validated endpoint polymerase chain reaction (PCR) assay for use in in-process and final product testing [8]. The authors received Health Canada approval to use this assay for our phase 1/2 MSC-osteoarthritis clinical trial (NCT02351011) [11,12]. In the previous study, autologous bone marrow-derived MSCs (MSCs(M)) were expanded in the authors' Good Manufacturing Practice (GMP) cell processing facility and tested, upon Health Canada approval, both in process and as part of final product release using the in-house qualified endpoint PCR mycoplasma kit.

Although endpoint PCR is faster than conventional methods, it has the following limitations: (i) it needs an additional post-PCR processing step such as gel electrophoresis to get final results; (ii) the results depend on band size-based discrimination and are not quantitative; (iii) it has a smaller dynamic range (<2 logs) and can detect only a range of about 10-fold (10 versus 100 copies); and (iv) it lacks a cell-type independent built-in control for DNA extraction efficiency [13,14].

To address these limitations, the authors investigated the use of a commercially available real-time PCR kit (MycotoOL mycoplasma real-time PCR kit; Roche Diagnostics GmbH, Mannheim, Germany), which has previously been validated by Roche according to European Pharmacopoeia (Ph. Eur.) 2.6.7 NAT validation guidelines for mycoplasma detection [15]. Real-time PCR is less time-consuming than endpoint PCR (no gel electrophoresis steps) and can detect 2-fold changes in gene expression, and the kit includes a cell-type independent recovery control (RC) as a control for DNA extraction and false-negative results [13–16].

In this study, the authors performed an abbreviated in-house qualification process similar to our previous efforts with a commercial endpoint PCR kit [8]. The authors leveraged the fact that Roche had fully validated the real-time PCR kit against nine mycoplasma species [15]. According to Ph. Eur. guidelines (section 2.6.7), the end user need not repeat the validation performed by the manufacturer. An abbreviated in-house qualification of the real-time PCR kit was performed focusing on limit of detection (LOD) using two commonly occurring mycoplasma genomic reference strains, *Mycoplasma arginini* (*M. arginini*) and *Mycoplasma hominis* (*M. hominis*) (American Type Culture Collection [ATCC], Manassas, VA, USA); ruggedness; and matrix interference testing. Additionally, the authors tested the use of a DNA carrier, polyadenylic acid (poly[A]), to facilitate the recovery of mycoplasma genomic DNA (gDNA) in diluted samples (<45 000 cells/mL), which posed a challenge in terms of efficient gDNA extraction. The addition of poly(A) did not cause any interference with mycoplasma gDNA isolation or amplification, suggesting it may be a useful approach for ensuring reliable real-time PCR results in samples with lower cell densities.

The authors demonstrated the fit-for-purpose utility of a commercially available real-time PCR kit for evaluating mycoplasma contamination in our previously used MSC(M) investigational product (NCT02351011) [11] and addressed poor recovery of mycoplasma gDNA in samples with low cell numbers (4500 and 45 000 cells/mL) with the use of poly(A) as a carrier during DNA extraction.

Methods

MSCs(M)

The study was approved by the University Health Network Institutional Ethics Board (REB20-5066) and conducted using human MSCs(M) banked from a previously conducted clinical trial (NCT02351011) [12]. Briefly, the MSCs(M) were expanded in Dulbecco's Modified Eagle's Medium, low glucose (Life Technologies, Carlsbad, CA, USA) supplemented with 1% GlutaMAX (Life Technologies) and 10% screened fetal bovine serum (HyClone; Cytiva, Marlborough, MA, USA) in a GMP cell manufacturing facility. Cryopreserved MSCs(M) were thawed and expanded in conditions mimicking their GMP expansion. MSCs(M) were harvested using TrypLE Express (Life Technologies). The pellet was washed once with Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies) and resuspended in DPBS. MSCs(M) and culture medium were tested to represent final product and in-process test samples. Similarly, the previous in-house qualification of the endpoint PCR kit was performed on representative final product samples [8]. However, this study was conducted as an independent qualification using the previous abbreviated qualification approach successfully approved by Health Canada; this is not intended to be a comparative study. In addition, to test the ability of the real-time PCR kit to detect mycoplasma in spent cell culture medium and to evaluate matrix interference from the cell culture medium, the authors performed additional experiments evaluating conditioned medium (CM) or cell culture supernatant spiked with mycoplasma gDNA.

Mycoplasma gDNA

MSCs(M) were spiked with gDNA standards from two species of mycoplasma: *M. hominis* (strain G230, qCRM-23838D; ATCC) and *M. arginini* (strain LBD-4, qCRM-27545D; ATCC). These are certified reference materials produced under International Organization for Standardization (ISO) Guide 34:2009 at ISO/IEC 17025:2005 and ISO 9001:2008 accredited/certified laboratories. The gDNA was used to avoid the need for additional laboratory biosafety assessments to accommodate live mycoplasma cultures. *M. arginini* and *M. hominis* were selected for the abbreviated qualification of in-house LOD for a direct comparison with the previous endpoint PCR qualification study [8]. In addition, *M. arginini* was selected because of its bovine tropism, as it may be a potential contaminant in fetal bovine serum, an ancillary agent used in expansion of MSCs(M) [12]. *M. hominis* has human tropism and may be a potential contaminant from operators [17,18]. ATCC provides genome copies (GCs)/μL in the certificate of analysis (CoA) accompanying the gDNA (Table 1). To meet the acceptance criteria of 10 colony-forming units (CFUs)/mL LOD [3], GC/mL to CFU/mL ratios were determined from the specific CoA and previously published data, specifically an average of 4 GCs/CFU for *M. arginini* [19] and an average of 24 GCs/CFU for *M. hominis* [20]. Based on this conversion ratio, gDNA from each mycoplasma species was diluted to obtain a dilution series ranging from 1 to 1000 CFUs/mL.

Table 1
Characteristics of mycoplasma species used in the study.

	Strain	ATCC number	Format supplied	Average titer	GCs/CFU	Origin	Prevalence, %
<i>M. arginini</i>	G230	qCRM-23838D	gDNA, frozen	6.4×10^6 GCs/μL	4 [19]	Bovine	20–30 [30]
<i>M. hominis</i>	LBD-4	qCRM-27545D	gDNA, frozen	5.2×10^6 GCs/μL	24 [20]	Human	10–20 [30]

The MSC(M) samples were spiked with mycoplasma gDNA to obtain final concentrations of 0.4 GC/mL (0.1 CFU/mL), 4 GCs/mL (1 CFU/mL), 40 GCs/mL (10 CFUs/mL) and 400 GCs/mL (100 CFUs/mL) of *M. arginini* and 2.4 GCs/mL (0.1 CFU/mL), 24 GCs/mL (1 CFU/mL), 240 GCs/mL (10 CFUs/mL) and 2400 GCs/mL (100 CFUs/mL) of *M. hominis* gDNA. For unspiked controls, DNA-free water was added and represented as 0 GCs/mL (0 CFUs/mL).

MycoTOOL mycoplasma real-time PCR kit

A MycoTOOL mycoplasma real-time PCR kit was used in conjunction with a manual DNA extraction kit (QC sample preparation kit; Roche Diagnostics GmbH, Mannheim, Germany). All reagents necessary for gDNA extraction and real-time PCR are included in these kits (see supplementary Table 1). Sample processing, master mix preparation and setup for PCR amplification were performed in spatially segregated areas.

Real-time PCR kit controls

The MycoTOOL mycoplasma real-time PCR kit includes several controls, including (i) an RC plasmid and (ii) a positive control (PC) plasmid. The RC plasmid is added to the test sample prior to DNA extraction. Positive amplification of the RC confirms the success and efficiency of DNA extraction. The RC may also help reveal PCR inhibition caused by matrix components. The PC reaction requires amplification of a plasmid template at a low copy number to verify the function of the PCR reagents.

Sample processing and gDNA extraction

MSCs(M) were harvested, washed and resuspended in DPBS (440 μ L). The RC plasmid (10 μ L) was added to the MSC(M) cell suspension together with *M. hominis* or *M. arginini* gDNA at various dilutions. These spiked MSC(M) samples were processed using the QC sample preparation kit for DNA extraction according to the manufacturer's instructions. The extracted DNA was used immediately or stored at -20°C for a maximum of 3 days.

DNA carrier

To improve mycoplasma gDNA recovery, a DNA carrier, poly(A) (supplied with the QC sample preparation kit), was added to the spiked MSC(M) samples during the lysis step at a final concentration of 0.2 mg/mL in the lysate. MSCs(M) were processed as previously described.

Detection of mycoplasma in the cell culture supernatant

The authors further investigated the ability of the kit to detect mycoplasma in a cell-free matrix. CM from MSC(M) cultures ≥ 48 h old was spiked with either *M. arginini* or *M. hominis* gDNA to yield a final GC/mL concentration equivalent to 10 CFUs/mL, as described earlier. Poly(A) (0.2 mg/mL) was added to facilitate DNA recovery, and the samples were processed as previously described. The authors used the spent medium or CM, as it represented an in-process cell-free sample.

Real-time PCR

Two separate PCR master mixes were prepared according to the kit instructions. The mycoplasma master mix contained primers and a fluorescein amidite-labeled detection probe for amplification and detection of mycoplasma gDNA and the PC plasmid. The RC master mix contained primers and a LightCycler yellow 555-labeled detection probe (Roche Diagnostics GmbH) for amplification and detection of the RC plasmid. MycoTOOL mycoplasma real-time PCR was performed in 96-well plates

(Roche Diagnostics Corporation) at a volume of 50 μ L/reaction according to the manufacturer's instructions. In each PCR plate, PC and test samples were run in technical replicates of four, with the no-template control in technical replicates of two. In each plate, five serial dilutions (GCs equivalent to 0 CFUs/mL, 0.1 CFU/mL, 1 CFU/mL, 10 CFUs/mL and 100 CFUs/mL) were used for *M. arginini* and four serial dilutions (GCs equivalent to 0 CFUs/mL, 1 CFU/mL, 10 CFUs/mL and 100 CFUs/mL) were used for *M. hominis*.

Data analysis

Data analysis was conducted with LightCycler 480 version 1.5.1.62 software (LC480; Roche Diagnostics International Ltd., Rotkreuz, Switzerland) using the absolute quantification /second derivative maximum method for calculation of crossing point (Cp) values. Fluorescence channels fluorescein amidite (465–510) and VIC/HEX/yellow 555 (533–580) were used for mycoplasma detection and RC detection, respectively. The LightCycler 480 software calculates Cp values for signal quantification, and a Cp cutoff value of 43.0 was used in this study. This is the default cutoff value under the instrument cycling conditions provided by the PCR kit manufacturer. All results with $\text{Cp} \geq 43.0$ are scored as negative. All test results were verified against the acceptance criteria for a valid test result (i.e., all PCs produced positive results and all negative controls produced negative results) (see supplementary Table 2). If the RC PCR replicates show one or more negative results, the run is considered invalid and needs to be repeated.

Limit of detection

MSCs(M) ($n = 3$ biological replicates and 4 technical replicates) were spiked with 10-fold mycoplasma gDNA dilutions (400 GCs/mL, 40 GCs/mL, 4 GCs/mL and 0.4 GC/mL of *M. arginini* and 2400 GCs/mL, 240 GCs/mL and 24 GCs/mL for *M. hominis*), and LOD was determined by the lowest mycoplasma gDNA dilution detected by real-time PCR. LOD was also determined without (–) and with (+) the addition of poly(A) during DNA extraction.

Ruggedness

Ruggedness was evaluated by analyzing the different PCR results on different days. The percent coefficient of variation (%CV) was calculated by dividing the standard deviation by mean Cp value and then multiplying by 100.

Matrix interference

Matrix interference was tested using 10-fold dilutions of MSCs(M) down to 4500 cells/mL in DPBS, with spent media analyzed separately for interference as an additional experiment. MSCs(M) ($n = 1$ donor) were serially diluted (neat/undiluted, 1/10 dilution and 1/100 dilution) before being spiked with mycoplasma gDNA and processed as before. Matrix interference may be indicated by a decrease in Cp value of more dilute samples in real-time PCR [21,22]. The same experiment was repeated ($n = 2$) by adding poly(A) to improve mycoplasma gDNA recovery in diluted samples (1/10 and 1/100) with MSC(M) concentrations less than 45 000 cells/mL.

Statistics

Data from individual experiments are presented as number of positive wells (i.e., Cp value < 43) or mean Cp value with standard deviation. Two-way analysis of variance for multiple comparison was performed with Prism 8.4.3 (GraphPad Software, San Diego, CA, USA)

Figure 1. Schematic diagram showing experimental plan for abbreviated qualification of a real-time PCR kit for (A) LOD and ruggedness and (B) matrix interference.

to compare the mean difference between groups. A mean difference with $P \leq 0.05$ was considered significant.

Results

Abbreviated qualification strategy

The authors conducted an abbreviated qualification of a commercially available real-time PCR kit to detect mycoplasma, evaluating LOD with only two mycoplasma species (*M. arginini* and *M. hominis*) as well as ruggedness to demonstrate reproducibility of the PCR method and matrix interference to demonstrate fit-for-purpose utility with the MSC (M) investigational product (NCT02351011) [11]. LOD was determined by spiking MSCs(M) from three different biological donors with serial dilutions of mycoplasma gDNA. Matrix interference experiments were performed using dilutions of MSC(M) samples from two different donors ($n = 2$). Previous matrix interference experiments without poly(A) that were inconclusive because of poor DNA recovery were limited to one donor. Subsequent matrix interference experiments (with the addition of poly[A]) were conducted with two lots of MSCs(M) (Figure 1). Ruggedness was tested by performing the LOD and matrix interference experiments on different days.

A total of 22 PCR runs were carried out on different days, a subset of which met the acceptance criteria for a valid test (see supplementary Table 3). These runs were used for generating data for LOD (six runs) and matrix interference (without poly[A], six runs; with poly[A], 12 runs) and for calculating ruggedness (18 runs).

Sensitivity/LOD

The kit manufacturer has validated the MycoTOOL mycoplasma real-time PCR kit for LOD against a panel of 10 mycoplasma species [15]. This exceeds the recommendation of seven species by Ph. Eur. [3] and five species by the US Pharmacopeia [4].

Data from six valid PCR runs (three runs each for *M. arginini* and *M. hominis*) were used to generate the required data for determining LOD. PC reactions were all positive (24 of 24, 6×4 technical replicates), with a mean Cp value of 34.08 ± 0.44 (%CV, 1.29), and no-template controls were all negative (zero of 12, 6×2 technical replicates) in all runs (see supplementary Table 3). RCs were positive for all samples (108 of 108, 3×5 dilutions $\times 4$ technical replicates for *M. arginini* and 3×4 dilutions $\times 4$ technical replicates for *M. hominis*), with a mean Cp = 38.08 ± 0.02 (%CV, 0.05). Since the kit did not detect *M. hominis* at 2.4 GCs/mL (0.1 CFU/mL) in pilot experiments (data not shown), this dilution was excluded. Similarly, *M. arginini* at 0.4 GC/mL (0.1 CFU/mL) was not detected by the kit and was excluded from further analysis.

For qualification purposes, the authors relied on a conservative 95% detection rate [7]. Under this stringent criterion, MSCs(M) ($n = 12$ PCR reactions, 3 biological $\times 4$ technical replicates) spiked with mycoplasma gDNA showed an LOD ≤ 40 GCs/mL (10 CFUs/mL) and ≤ 240 GCs/mL (10 CFUs/mL) for *M. arginini* and *M. hominis*, respectively (Table 2). However, the manufacturer criterion considers one of four wells (technical replicates) to represent a positive result. Using this criterion, wells were positive for mycoplasma when spiked with 0.4 GC/mL (0.1 CFU/mL, one of 12, 8% detection rate) for *M. arginini* and 24 GCs/mL (1 CFU/mL, six of 12, 50% detection rate) for *M. hominis*. Additionally, for *M. arginini*, there was heterogeneity among MSC(M) biological donors, with one donor demonstrating an LOD of 4 GCs/mL (100% detection rate), whereas the other two demonstrated an LOD of 40 GCs/mL (100% detection rate) by conservative criteria.

Matrix interference

The difference in mean Cp value over 10-fold dilutions of MSC(M) samples spiked with mycoplasma gDNA was used to assess the presence of matrix interference. Matrix interference may be indicated by a decrease in Cp value in diluted versus undiluted samples. Initial

Table 2

Summary of mycoplasma PCR reactions demonstrating LOD with two mycoplasma species in undiluted BM-MSCs samples.

	Number of positive reactions/number of total reactions			
	BM-MSCs, donor #1 ^a	BM-MSCs, donor #2 ^a	BM-MSCs, donor #3 ^a	Σ (% positive reactions)
<i>M. arginini</i>				
400 GCs/mL (100 CFUs/mL)	4/4	4/4	4/4	12/12 (100)
40 GCs/mL (10 CFUs/mL)	4/4	4/4	4/4	12/12 (100)
4 GCs/mL (1 CFU/mL)	1/4	1/4	4/4	6/12 (50)
0.4 GC/mL (0.1 CFU/mL)	0/4	0/4	1/4	1/12 (8)
0 GCs/mL (0 CFUs/mL)	0/4	0/4	0/4	0/12 (0)
<i>M. hominis</i>				
2400 GCs/mL (100 CFUs/mL)	4/4	4/4	4/4	12/12 (100)
240 GCs/mL (10 CFUs/mL)	4/4	4/4	4/4	12/12 (100)
24 GCs/mL (1 CFU/mL)	3/4	3/4	0/4	6/12 (50)
2.4 GC/mL (0.1 CFU/mL) ^b	NA	NA	NA	NA
0 GCs/mL (0 CFUs/mL)	0/4	0/4	0/4	0/12 (0)

Biological replicates, $n = 3 \times 4$ technical replicates; these reactions were carried out without poly(A).

NA, not applicable; NTCs, no-template control.

^a Biological replicates.

^b 2.4 GC/mL (0.1 CFU/mL) spiking concentration was omitted, as preliminary experiments did not detect *M. hominis* gDNA at this concentration; data not included, as the run was marked invalid as a result of NTCs showing a Cp value of 43 (cutoff, Cp ≥ 43).

Table 3

Summary of mycoplasma PCR reactions at different dilutions of BM-MSCs without (–) and with (+) poly(A) to test matrix interference.

	Number of positive reactions/number of total reactions								
	Undiluted BM-MSCs (450 000 cells/mL)			1/10 diluted BM-MSCs (45 000 cells/mL)			1/100 diluted BM-MSCs (4500 cells/mL)		
	BM-MSCs, donor #1	BM-MSCs, donor #2	Σ (% positive reactions)	BM-MSCs, donor #1	BM-MSCs, donor #2	Σ (% positive reactions)	BM-MSCs, donor #1	BM-MSCs, donor #2	Σ (% positive reactions)
(–) Poly(A) (n = 1) ^a									
<i>M. arginini</i>									
400 GCs/mL (100 CFUs/mL)	4/4	NA	4/4 (100)	1/4	NA	1/4 (25)	2/4	NA	2/4 (50)
40 GCs/mL (10 CFUs/mL)	4/4	NA	4/4 (100)	2/4	NA	2/4 (50)	1/4	NA	1/4 (25)
4 GCs/mL (1 CFU/mL)	1/4	NA	1/4 (25)	0/4	NA	0/4 (0)	0/4	NA	0/4 (0)
0 GCs/mL (0 CFUs/mL)	0/4	NA	0/4 (0)	0/4	NA	0/4 (0)	0/4	NA	0/4 (0)
<i>M. hominis</i>									
2400 GCs/mL (100 CFUs/mL)	4/4	NA	4/4 (100)	4/4	NA	4/4 (100)	3/4	NA	3/4 (75)
240 GCs/mL (10 CFUs/mL)	4/4	NA	4/4 (100)	0/4	NA	0/4 (0)	0/4	NA	0/4 (0)
24 GCs/mL (1 CFU/mL)	3/4	NA	3/4 (75)	0/4	NA	0/4 (0)	0/4	NA	0/4 (0)
0 GCs/mL (0 CFUs/mL)	0/4	NA	0/4 (0)	0/4	NA	0/4 (0)	0/4	NA	0/4 (0)
(+) Poly(A) (n = 2) ^b									
<i>M. arginini</i> + poly(A)									
400 GCs/mL (100 CFUs/mL)	4/4	4/4	8/8 (100)	4/4	4/4	8/8 (100)	4/4	4/4	8/8 (100)
40 GCs/mL (10 CFUs/mL)	4/4	4/4	8/8 (100)	4/4	4/4	8/8 (100)	3/4	4/4	7/8 (88)
4 GCs/mL (1 CFU/mL)	0/4	1/4	1/8 (13)	1/4	1/4	2/8 (25)	1/4	1/4	2/8 (25)
0 GCs/mL (0 CFUs/mL)	0/4	0/4	0/8 (0)	0/4	0/4	0/8 (0)	0/4	0/4	0/8 (0)
<i>M. hominis</i> + poly(A)									
2400 GCs/mL (100 CFUs/mL)	4/4	4/4	8/8 (100)	4/4	4/4	8/8 (100)	4/4	4/4	8/8 (100)
240 GCs/mL (10 CFUs/mL)	4/4	4/4	8/8 (100)	4/4	4/4	8/8 (100)	4/4	4/4	8/8 (100)
4 GCs/mL (1 CFU/mL)	3/4	3/4	6/8 (75)	3/4	4/4	7/8 (88)	4/4	3/4	7/8 (88)
0 GCs/mL (0 CFUs/mL)	0/4	0/4	0/8 (0)	0/4	0/4	0/8 (0)	0/4	0/4	0/8 (0)

NA, not applicable.

^a Biological replicate, n = 1 × 4 technical replicates; these reactions were carried out without poly(A).^b Biological replicates, n = 2 × 4 technical replicates; these reactions were carried out with poly(A).

experiments (without the addition of poly[A], six runs) (see supplementary Table 3) failed to detect lower concentrations of spiked-in mycoplasma gDNA (<40 GCs/mL for *M. arginini* and <2400 GCs/mL for *M. hominis*) in diluted MSC(M) samples (1/10 or 45 000 cells/mL, 1/100 or 4500 cells/mL) in all tested conditions, hindering the matrix interference analysis (Table 3, Figure 2A,B). The experiment also failed to achieve the required LOD, considered here to be equivalent to 10 CFUs/mL, in the diluted samples (Table 3). Since the available data for mycoplasma and RC PCR did not show any decrease in Cp values in 1/10 (45 000 cells/mL) and 1/100 (4500 cells/mL) dilutions, the authors inferred that there was no matrix interference (Figure 2A,B; also see supplementary Table 4). However, because the authors did not obtain valid Cp values for many of the diluted MSC (M) samples, it was reasoned that this was due to lower gDNA recovery. To address this issue, the authors looked at strategies to improve gDNA recovery.

Addition of poly(A)

A carrier, poly(A), was added to facilitate improved DNA recovery of mycoplasma gDNA in samples with low starting MSC(M) cell numbers (diluted samples, 1/10 dilution or 45 000 cells/mL and 1/100 dilution or 4500 cell/mL). Poly(A) aids in DNA recovery by binding to DNA during precipitation and also provides a larger and more visible pellet, reducing the possibility of DNA pellet loss during extraction in low cell density samples. A total of 12 PCR runs were carried out with poly(A) (see supplementary Table 3).

The addition of poly(A) resulted in improved mycoplasma gDNA recovery and enabled an LOD, considered here to be equivalent to 10 CFUs/mL, to be attained at 1/10 (45 000 cell/mL) and 1/100 (4500 cell/mL) dilutions for both *M. arginini* and *M. hominis* (Table 3, Figure 2C,D). Achieving an LOD, considered here to be equivalent to 10 CFUs/mL, was not possible in diluted MSC(M) samples in the absence of poly(A) (Table 3).

By contrast, the addition of poly(A) did not affect the mean Cp value of mycoplasma or RC PCR or the LOD in undiluted MSC(M)

samples (450 000 cells/mL) (Figure 3). LOD without and with poly(A) was equivalent to 10 CFUs/mL for *M. arginini* (40 GCs/mL) and *M. hominis* (240 GCs/mL) in undiluted MSC(M) samples (Table 3).

The addition of poly(A) did not produce any significant decrease ($P > 0.05$) in mycoplasma PCR mean Cp values for diluted MSC(M) samples (1/10 dilution or 45 000 cells/mL, 1/100 dilution or 4500 cells/mL) compared with undiluted MSC(M) samples (450 000 cells/mL) across the *M. arginini* and *M. hominis* gDNA dilutions (Figure 2C,D). This confirmed the definitive absence of sample matrix interference, which could not be confirmed in the absence of poly(A).

In addition to mycoplasma PCR, the authors analyzed RC PCR Cp values from the matrix interference experiments with poly(A). Diluted MSC(M) samples (4500–45 000 cells/mL) spiked with various concentrations of *M. hominis* and *M. arginini* gDNA did not show any significant change in RC PCR Cp values when compared with undiluted or neat MSC(M) samples (450 000 cells/mL) (see supplementary Figure 1; see supplementary Table 4).

Additionally, the authors analyzed CM from MSC(M) samples spiked with mycoplasma gDNA and processed with and without poly (A) and compared the Cp values with washed MSCs(M) in DPBS spiked with mycoplasma gDNA. The authors noted no change in mean Cp values at the LOD, considered here to be equivalent to 10 CFUs/mL, for *M. arginini* and *M. hominis* gDNA (see supplementary Table 5), indicating that CM as well as the addition of poly(A) does not, in and of itself, cause any matrix interference.

Ruggedness

From a total of 22 valid PCR runs, data from 18 PCR runs (nine runs each for *M. arginini* and *M. hominis*) were used to assess ruggedness. Four runs with diluted MSC(M) samples (1/10 and 1/100) without poly(A) gave inconsistent results because of low cell numbers ($\leq 45 000$ cells/mL) and poor gDNA recovery and were therefore omitted.

Analysis of mycoplasma PCR Cp values showed an inter-assay %CV <4 and an intra-assay %CV <3 for both *M. arginini* and *M. hominis*

Figure 2. Addition of poly(A) improves gDNA recovery in diluted BM-MSC samples (4500–45 000 cells/mL). (A,B) Mean Cp values for the matrix interference assay using 10-fold dilutions of BM-MSCs without (–) poly(A) show poor mycoplasma gDNA recovery, as indicated by higher mean Cp values in diluted (1/10, 1/100) BM-MSC samples. Data are represented as mean $\pm$ SD ($n = 1$ biological replicate $\times$ 4 technical replicates). (C,D) Addition of poly(A) improves mycoplasma gDNA recovery, as indicated by the lack of change in mean Cp values across the dilution of BM-MSC samples (two-way ANOVA with multiple comparisons, *M. arginini* $P = 0.60$ and *M. hominis* $P = 0.10$). Dotted line shows the cutoff Cp value. Data are represented as mean $\pm$ SD ($n = 2$ biological replicates $\times$ 4 technical replicates). ● = neat/undiluted BM-MSCs, 450 000 cells/mL; ■ = 1/10 diluted BM-MSCs, 45 000 cells/mL; ▲ = 1/100 diluted BM-MSCs, 4500 cells/mL. ANOVA, analysis of variance; SD, standard deviation.

(Table 4). Similarly, for RC PCR Cp values, all runs showed a %CV <3 for both *M. arginini* (0.40–2.95) and *M. hominis* (0.21–1.44) (see supplementary Table 6). A %CV <5 across all runs indicated reliable and reproducible performance of the assay.

Discussion

The authors previously reported an abbreviated in-house qualification of an endpoint PCR kit for mycoplasma detection in MSC (M) therapy products [8]. In this study, the authors applied the same abbreviated qualification strategy to a real-time PCR kit using the same MSC(M) samples ($n = 3$ donors) spiked with gDNA from two mycoplasma species. The abbreviated qualification included assessments of LOD for the two mycoplasma species as well as ruggedness and matrix interference. Mycoplasma gDNA recovery was reduced as cell numbers decreased, but recovery was enhanced by the addition of a poly(A) to low cell density samples. The addition of poly(A) did not improve LOD for undiluted MSC(M) samples, which had sufficient numbers of starting cells (≥ 45 000 cells/mL). However, it did improve LOD for diluted samples (≤ 45 000 cells/mL). Importantly, poly(A) did not itself cause any interference either with or without cells. The data shown in this study are from the experiments performed by a single operator, each step verified by a second analyst. Since this was an abbreviated qualification study, the authors followed this approach with a single operator, conducting the PCR runs on different days, with verification by secondary analysts. LOD and matrix interference tests done on different days by the same operator resulted in %CV <4. The MycoTOOL mycoplasma real-time PCR kit was qualified in-house and showed comparable

sensitivity to the MycoTOOL endpoint PCR kit, reduced assay time (<6 h) and comparable costs [8].

NAT-based methods assess mycoplasma contamination via detection of DNA arising from viable, non-viable and lysed microorganisms. Laboratories without facilities to grow live mycoplasma cultures often depend on published GC/CFU conversion ratios, which are highly variable and depend on the strain of mycoplasma and methods used for the culture and quantification of the GCs [7,8]. As there are no standard GC/CFU conversion ratios, applying this conversion in practice has challenges. For the real-time PCR qualification study, the authors used GCs/mL instead of GCs/reaction to calculate a GC/mL equivalent to 10 CFUs/mL. Based on the published GC/CFU ratios [19,20], 10 CFUs/mL is equivalent to 40 GCs/mL and 240 GCs/mL of *M. arginini* and *M. hominis* gDNA, respectively. This is different from the authors' previous in-house endpoint PCR qualification article, which used 18 GCs/reaction and 107 GCs/reaction for *M. arginini* and *M. hominis*, respectively [8]. In the authors' endpoint PCR qualification, the required GCs/reaction to achieve the LOD, considered here to be equivalent to 10 CFUs/mL, was calculated based on a 450- μ L test sample volume rather than a PCR reaction volume. Using an alternative method for counting the volume of the PCR reaction (50 μ L) instead of the volume of the sample (450 μ L) would result in 2 GCs/reaction for *M. arginini* and 11.9 GCs/reaction for *M. hominis*, which is 9-fold lower than the GCs/reaction previously calculated for endpoint PCR.

For real-time PCR, 450 μ L of MSCs(M) spiked with mycoplasma gDNA was processed for DNA extraction, and the extracted DNA was eluted in 200 μ L of buffer, of which 20 μ L was used in each 50- μ L PCR reaction volume, which means that only 10% of the extracted DNA was used in each PCR reaction. However, it is not possible to extract the spiked-in mycoplasma gDNA with 100% efficiency, and therefore the number of

Figure 3. Addition of poly(A) carrier does not affect mean mycoplasma or RC PCR Cp values of undiluted BM-MSc samples (450 000 cells/mL). (A,B) Addition of poly(A) does not change mean mycoplasma PCR Cp values, with the exception of BM-MSCs spiked with 2400 GCs/mL of *M. hominis* (two-way ANOVA with multiple comparisons, -1.44 cycles, $P = 0.002$). BM-MSCs spiked with 4 GCs/mL of *M. arginini* and processed without poly(A) showed a mean Cp value of 39.8 (six of 12, 50% positivity) compared with a Cp value of 42.6 (one of eight, 13% positivity) in BM-MSCs processed with poly(A). Since the positivity rate was low, this observation was not considered significant. (C) BM-MSc samples spiked with various concentrations of *M. arginini* gDNA show no significant change in RC Cp values ($P > 0.05$) with the addition of poly(A). (D) BM-MSc samples spiked with 2400 GCs/mL and 240 GCs/mL of *M. hominis* gDNA do not show any significant change in RC Cp values ($P > 0.05$), whereas BM-MSc samples spiked with 24 GCs/mL (1 CFU/mL, 0.87 cycle, $P = 0.03$) and unspiked samples (0 GCs/mL, 0 CFUs/mL, 0.82 cycle, $P = 0.04$) show an increase in Cp values (two-way ANOVA with multiple comparisons). However, these differences were seen <2 cycles. The dotted line shows the cutoff Cp value. Data are represented as mean $\pm$ SD (biological replicates, $(-)$ poly(A), $n = 3$, $(+)$ poly(A), $n = 2$). * $P < 0.05$. $\bullet = (-)$ poly(A), BM-MSCs processed without poly(A); $\blacksquare = (+)$ poly(A), BM-MSCs processed with poly(A). ANOVA, analysis of variance; SD, standard deviation.

GCs in PCR will be lower than the required CFU/mL threshold. For this reason, for real-time PCR, the authors calculated and expressed the LOD in terms of GCs/mL rather than GCs/reaction.

The sensitivity of NAT-based assays varies depending on the type of assay method, sample source, cell number and, most importantly, species and reference strain of mycoplasma used [14,15,19,20,22]. Roche's validation data for the real-time PCR kit using 1 mL of standard Chinese hamster ovary cell culture show an LOD of 0.3 CFU/mL and 0.2 CFU/mL for *M. arginini* (ATCC23838) and *M. hominis* (ATCC23114), respectively [15]. However, the current abbreviated real-time PCR qualification study did not demonstrate an LOD <10 CFUs/mL for either *M. arginini* or *M. hominis*. Apart from using a different cell type (MSCs(M)), this could be because the authors used mycoplasma gDNA rather than live mycoplasma for spiking and therefore depended on a published GC/CFU ratio [19,20] for calculations. Interestingly, under similar testing conditions, the real-time PCR method demonstrated a more conservative LOD (10 CFUs/mL) compared with the authors' previously published endpoint PCR LOD (<10 CFUs/mL) [8].

MSC(M) samples with low cell numbers (<45 000 cells) sometimes resulted in loss of the DNA pellet during processing. The observed variable DNA recovery was probably due to smaller size and lower amount of cellular DNA (resulting from reduced cell concentrations) available to bind to spiked-in mycoplasma gDNA and RC plasmid DNA. This, together with the use of more diluted gDNA concentrations, likely resulted in reduced recovery of mycoplasma gDNA. The addition of carrier nucleic acids increases the yield of extracted DNA/RNA from samples low in cell numbers or DNA/RNA concentration [23]. Poly(A), MS2 RNA and transfer RNA from yeast are some of the commonly used carriers in nucleic acid extractions

from clinical or experimental samples [23–26]. The authors' matrix interference results suggest that poly(A) can be used with low sample cell numbers (≤ 45 000 cells/mL) to increase mycoplasma gDNA recovery and improve LOD without matrix interference. This is a useful strategy for preserving low sample volumes for testing investigational cell products for mycoplasma contamination.

The authors have qualified the real-time PCR kit for in-house testing for mycoplasma contamination in investigational MSC(M) products. In process, MSCs(M) and culture medium represent dual sources of matrix interference. With regard to the final product, MSCs(M) washed three times and resuspended in a neutral buffer such as DPBS represent the source of matrix interference. For analyzing matrix interference in this qualification study, the authors therefore used MSCs(M) washed once in DPBS, representative of both an in-process and final product, and the spent culture medium, representative of an in-process cell-free sample. This allowed the authors to separately conclude the absence of matrix interference from either the cells or the culture medium. However, the authors did not do both together, as this was an abbreviated qualification study and the authors wanted to limit the number of test conditions.

Another limitation of this study is the use of mycoplasma gDNA for spiking instead of live or inactivated mycoplasma. NAT method qualifications using samples spiked with mycoplasma gDNA have been previously reviewed and accepted by Health Canada [8,27]. Additionally, as the authors wanted to leverage and compare our qualification of the real-time PCR method with our previous qualification of an endpoint PCR kit for the same MSC(M) investigational cell product [8], the authors wanted to stay with the same approach of using gDNA.

This study did not account for lot-to-lot variability of mycoplasma gDNA, as the authors employed only one representative lot of

Table 4
Intra-assay and inter-assay %CV of Cp values for mycoplasma PCR for demonstrating ruggedness.

Run	100 CFU/mL equivalent				10 CFU/mL equivalent				1 CFU/mL equivalent			
	Mean Cp	SD	%CV, intra-assay (n = 4) ^a	%CV, inter-assay (n = 9) ^b	Mean Cp	SD	%CV, intra-assay (n = 4) ^a	%CV, inter-assay (n = 9) ^b	Mean Cp	SD	%CV, intra-assay (n = 4) ^a	%CV, inter-assay (n = 8) ^b
<i>M. arginini</i>												
1	36.27	0.48	1.32	3.62	40.39	1.57	3.89	3.61	39.51	1.00	2.53	NA
2	36.51	0.51	1.40		39.32	0.20	0.51		40.18	NA	NA	
3	32.12	0.77	2.40		35.86	2.06	5.74		40.47	NA	NA	
4 ^c	36.21	0.17	0.47		39.85	0.69	1.73		ND	NA	NA	
5 ^c	36.31	0.51	1.40		41.15	1.43	3.48		39.66	NA	NA	
6 ^c	36.07	0.32	0.89		39.81	1.17	2.94		41.00	NA	NA	
7 ^c	35.89	0.39	1.09		40.35	1.60	3.97		42.60	NA	NA	
8 ^c	35.32	0.40	1.13		40.29	1.88	4.67		41.18	NA	NA	
9 ^c	36.25	0.45	1.24		40.20	1.43	3.56		40.93	NA	NA	
<i>M. hominis</i>												
1	33.97	0.01	0.03	2.83	37.53	0.26	0.69	1.95	ND	NA	NA	1.49
2	33.36	0.14	0.42		36.71	0.53	1.44		40.32	0.68	1.69	
3	35.84	0.69	1.93		38.69	0.16	0.41		41.23	1.54	3.74	
4 ^c	33.46	0.16	0.48		36.64	0.42	1.15		39.88	0.78	1.96	
5 ^c	33.17	0.09	0.27		37.28	0.5	1.34		40.42	0.84	2.08	
6 ^c	33.06	0.29	0.88		36.78	0.41	1.11		39.29	0.75	1.91	
7 ^c	32.76	0.15	0.46		36.63	0.32	0.87		39.42	0.37	0.94	
8 ^c	32.46	0.11	0.34		36.05	0.23	0.64		39.67	0.63	1.59	
9 ^c	32.84	0.21	0.64		36.57	0.27	0.74		40.37	0.24	0.59	

NA, not applicable; ND, not determined; SD, standard deviation.

^a n = number of technical replicates.

^b n = number of PCR runs.

^c PCR runs with poly(A).

mycoplasma gDNA and relied on the manufacturer's CoA for the GCs/uL, which is yet another limitation. The authors used a previously published GC/CFU ratio (Table 1) that is representative of some lot preparations of *M. arginini* (4 GCs/CFU [19]) and *M. hominis* (24 GCs/CFU [20]). Lot-to-lot differences in GC/CFU ratios might occur, which were not accounted for in this study, and likely influenced the authors' ability to achieve a lower LOD based on these GC/CFU calculations. Spiking the MSCs(M) with live mycoplasma would have the advantage of mimicking real contamination. In addition, it gives the opportunity for parallel comparison of both NAT-based assay and compendial methods in a qualification study, which is more meaningful when replacing the conventional method with an alternative real-time PCR method [28]. However, using mycoplasma gDNA instead of live cultures expedites the method qualification, as it is easier to perform and less time-consuming and does not require special mycoplasma culture facilities.

This qualification study was done specifically for MSCs(M), the same investigational cell product that was used in the authors' previous Health Canada-approved endpoint PCR kit qualification [8] to leverage the qualification data and strategy for regulatory approval. Recent studies have also reported NAT method qualification for mycoplasma detection in other therapeutic cell products, such as chimeric antigen receptor T cells [27] and Epstein–Barr virus-specific cytotoxic T lymphocytes [29].

Conclusions

The abbreviated qualification of the MycoTOOL mycoplasma real-time PCR kit suggests it can conservatively detect 10 CFUs/mL for two commonly found mycoplasma species (*M. arginini* and *M. hominis*) and up to 1 CFU/mL using manufacturer-based criteria. The addition of a poly(A) carrier improves gDNA extraction and enables LOD, considered here to be equivalent to 10 CFUs/mL, in diluted samples with low cell numbers ($\leq 45,000$ cells/mL). The kit is minimally qualified for use with an investigational MSC(M) product (NCT02351011), demonstrates no matrix interference and, although not vetted by regulators, is likely to be acceptable based on previous experience with endpoint PCR in-house qualification [8].

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: SV, SM and RR. Acquisition of data: SM, RR and MR. Analysis and interpretation of data: SM, SV and RR. Drafting or revising the manuscript: SM and SV. All authors have approved the final article.

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2021.07.002.

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