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Optimizing resazurin-based viability assays for P-MSC/TER308 cell line to enhance results reliability

Jessica Petiti[1*], Laura Revel[1] and Carla Divieto[1]

Abstract

Objective The results of this research contribute to the LifeSaver project, which focuses on reducing neonatal and infant mortality resulting from preterm births. The project aims to create an in vitro system simulating prenatal conditions to screen and analyze chemicals and pharmaceuticals, establishing scientifically justified regulations for their use during pregnancy. Because several papers have recently identified data inconsistencies in pre-clinical studies, a key part of the project involves optimizing cellular cytotoxicity assays to enhance the reliability of pharmacological and toxicity screening for drugs and environmental contaminants.

Results The resazurin-based viability assay was chosen as the primary method due to its widespread adoption and simplicity in assessing drug cytotoxicity. This work describes the optimization of the resazurin-based viability assay on the P-MSC/TERT308 cell line, a placenta-derived mesenchymal stem cell used within the LifeSaver project. By applying our previously described and validated Standard Operating Procedure, we fine-tuned experimental parameters, consistently obtaining reliable results with measurement uncertainty of less than 10%.

Keywords Cytotoxicity assay, Resazurin-based viability assay, P-MSC/TERT308 cell line, Pharmacological and toxicity screening, Reliability of results

Introduction

In Europe, a significant percentage of neonatal and infant deaths are attributed to preterm births, with approximately 75% of neonatal deaths and 60% of infant deaths occurring in preterm infants [1]. The rising number of preterm births is influenced by factors such as advanced maternal age, environmental threats, and limited suitable treatments [1].

The LifeSaver project [2] aims to support every pregnant woman to have a safe living environment, protected by scientifically justified regulations governing the use and control of potentially harmful chemical and medicinal products. The project objective is to create a digitally cloned in vitro system that emulates prenatal conditions near the uterine/placental interface, contributing with a scientifically-based tool for screening and analyzing chemicals and pharmaceuticals, aiding in risk classification, regulations, and risk assessment/mitigation measures for pregnant women and fetal health.

In this context, it is crucial to note the recent emphasis on the presence of data inconsistencies in pre-clinical studies [3, 4]. This inconsistency hampers result comparisons and raises concerns about the reliability of findings.

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The choice of cell lines introduces variability that can impact the repeatability of results [5]. Experimental protocols, from reagent concentrations to assay conditions, must be standardized to mitigate inconsistencies [5].

To improve the reliability of pharmacological and toxicity screening for drugs and environmental contaminants, as a part of this ambitious project, efforts are dedicated to optimize cellular cytotoxicity tests. To this end, the resazurin-based assay has been selected as the primary method. Indeed, the resazurin-based viability assay, also known as Alamar blue, is one of the most adopted tools to assess drug cytotoxicity [6]. Exploiting the ability of living cells to reduce the non-fluorescent dye resazurin into fluorescent resorufin, this assay provides a reliable indicator of cellular metabolic activity and, consequently, cell viability [6]. The simplicity and sensitivity of the resazurin assay make it particularly advantageous for assessing the cytotoxic effects of various substances on several cell lines [7]. Its versatility extends to high-throughput screening applications, enabling the rapid evaluation of several compounds and concentrations [5, 8].

With the goal to enhance the reliability of cytotoxicity results, this research note described the optimization of resazurin-based viability assay for a placenta-derived mesenchymal stem cell line (P-MSC/TERT308) employed in the LifeSaver project, by applying our previously established and validated Standard Operating Procedure (SOP) [9, 10].

Cell culture conditions and resazurin working solution Preparation

P-MSC/TERT308 cells (CHT-051-0308) were kindly provided by Evercyte GmbH and cultured following the manufacturer's recommended protocols [11]. Resazurin sodium salt (Invitrogen, Thermo Fisher Scientific) was dissolved in milliQ-H_2O, sterilized using a 0.22 µm filter, and stored at -20 °C. To minimize fluorescence intensity (FI) variability, a 44 µM resazurin Working Solution (WS) was freshly prepared in the complete cell medium, avoiding degradation by limiting exposure to light and temperature fluctuations. The stability of resazurin in these conditions was verified, showing no significant degradation over the assay duration.

λ_{Ex}-λ_{Em} and incubation time optimization

To optimize the excitation (Ex) and emission (Em) wavelength (λ) for the resazurin assay on the P-MSC/TERT308 cell line, we evaluated different combinations of λ_{Ex} and λ_{Em} in the resazurin spectra across 3 cell confluences: approximately 3×10^4 cells/cm² (high), 1.5×10^4 cells/cm² (medium), and 5×10^3 cells/cm² (low). Cells were detached following the manufacturer's recommendations, counted using a Neubauer chamber, diluted in complete medium, and seeded in triplicate into a pre-coated 96-well plate. After overnight (ON) incubation under standard culture conditions to ensure firm attachment to the plate, the culture medium was removed, and 100 µL of resazurin WS was added to each well, including triplicate Blank (resazurin WS only) wells. Cells were incubated for 4 h (h) under standard conditions, and the metabolized resazurin WS was transferred to a black 96-well plate for FI detection, using a Spark multimode microplate reader (Tecan) at 12 λ_{Ex}-λ_{Em} combinations, pairing 4 λ_{Ex} (530, 535, 540, and 545 nm) with 3 λ_{Em} (585, 590, and 595 nm).

To ensure uniform dye distribution, FI readings were performed after gently shaking the plate before each acquisition. The linearity of FI results was assessed for all combinations, showing high R^2 values (0.990–0.999, mean: 0.997 ± 0.003). To determine the optimal λ_{Ex}-λ_{Em} combination, we analyzed the FI differences between experimental wells and Blank ($FI_{Sample-Blank}$) for each confluence and assigned scores accordingly: a higher $FI_{Sample-Blank}$ difference corresponded to a higher score. Summing these scores across all confluences, we identified 2 top λ_{Ex}-λ_{Em} combinations: 540–585 nm and 535–590 nm (Fig. 1A). While the λ_{Ex} of 540 nm and 535 nm were approximately equivalent, the λ_{Em} of 590 nm exhibited lower background noise compared to 585 nm. Therefore, we selected 535 nm for excitation and 590 nm for emission as the optimal λ_{Ex}-λ_{Em} combination for the resazurin assay on the P-MSC/TERT308 cell line.

Then, to identify the optimal incubation time, P-MSC/TERT308 cells were detached and counted as previously described. Three cell confluences, ranging from very low ($\sim 2 \times 10^2$ cells/cm²) to very high ($\sim 3.5 \times 10^4$ cells/cm²) were prepared in the complete medium and seeded into a pre-coated 96-well plate. For each incubation time point (1, 2, 4, and 6 h), triplicate wells were prepared, including Blank. After ON incubation under standard culture conditions to ensure firm attachment to the plate, the culture medium was removed, and 100 µL of resazurin WS was added to each well. Cells were incubated under standard conditions and, at each time point, metabolized resazurin WS was transferred to a black 96-well plate for FI detection using the Spark multimode microplate reader at λ_{Ex} 535 nm and λ_{Em} 590 nm.

The optimal incubation time for P-MSC/TERT308 cells treated with 44 µM resazurin WS was determined by evaluating the linearity of FI data. Results indicated that 6 h is optimal for cell concentrations from $\sim 4 \times 10^2$ to 2×10^3 cells/cm², 4 h for 2×10^3 to 1.7×10^4 cells/cm², and 2 h for 1.7×10^4 to 3.5×10^4 cells/cm² (Fig. 1B). Prolonged incubation led to a plateau in the FI curve, attributed to the loss of direct correlation between resazurin reduction and viable cell numbers. For experiments covering a wide range of concentrations, a 4-h incubation provided

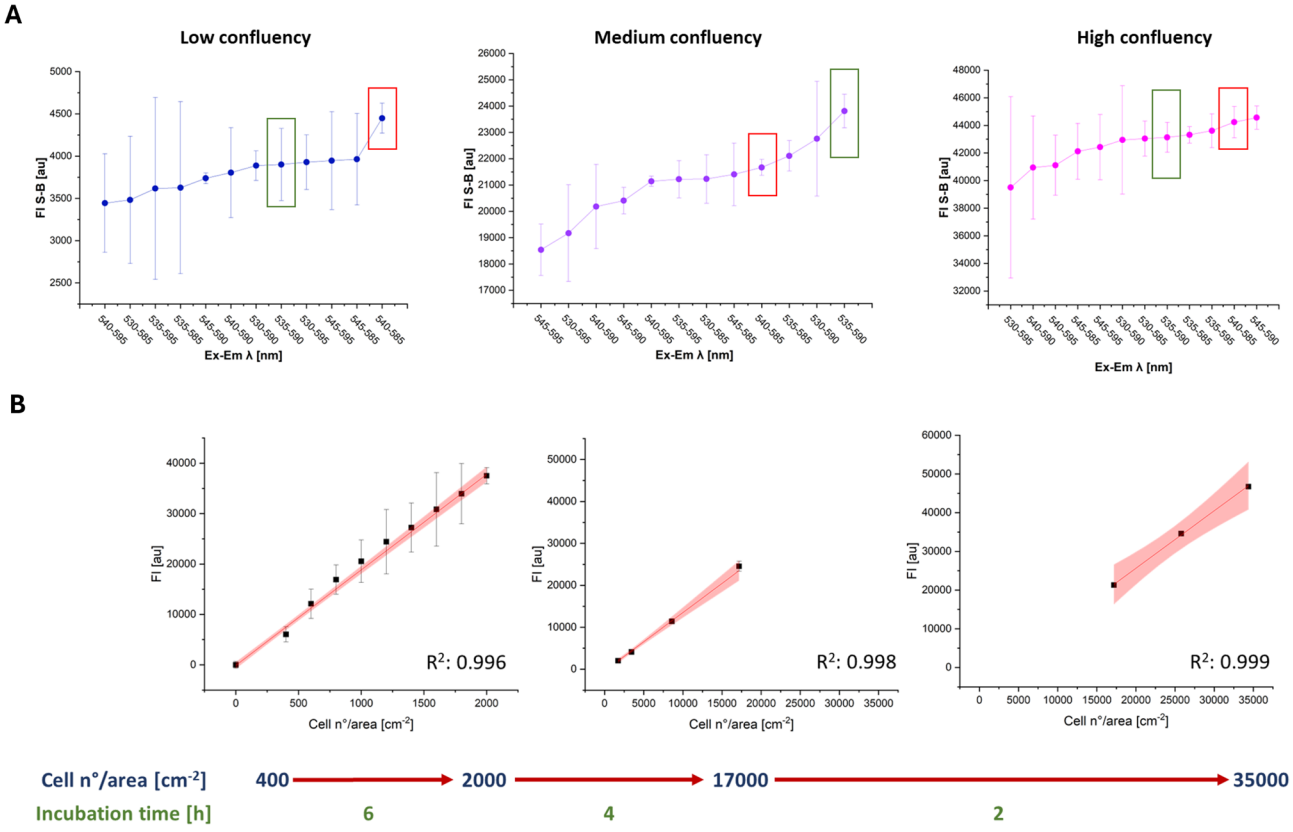

Fig. 1 **(A)** Fluorescence intensity difference between experimental wells and the Blank sample (FI S-B, *y*-axis) versus Excitation-Emission wavelengths (Ex-Em λ) combinations (*x*-axis). FI S-B is expressed in arbitrary units (au); (λEx-λEm) is expressed in nanometers (nm); error bars indicate the standard deviation (*SD*). The analysis is categorized according to cell confluency levels: low, medium, and high. Two distinct λ_{Ex}-λ_{Em} combinations have been identified as suitable and are highlighted within boxes: 540–585 nm (red box) and 535–590 nm (green box). **(B)** $FI_{Sample-Blank}$ (*y*-axis) plotted against cell concentration (*x*-axis). Fluorescence intensity (FI) is expressed as arbitrary units (au), and error bars represent standard deviation (*SD*). Linearity is indicated by R^2 values. The recommended incubation time is indicated in green respect to the cell concentration range (blue)

reliable results (R^2: 0.998) within $\sim 1.7 \times 10^3$ to $\sim 3.5 \times 10^4$ cells/cm².

Limit of blank, limit of detection, and limit of quantification

P-MSC/TERT308 cells were detached and counted as previously described to perform a very low confluency curve analysis, consisting of 12 data points ranging from $\sim 1 \times 10^2$ to 2×10^3 cells/cm². This analysis aimed to estimate the Limit of Blank (LoB) [12], Limit of Detection (LoD) [13], and Limit of Quantification (LoQ) [14]. Cells were diluted in the complete medium and seeded in triplicate into a pre-coated 96-well plate. After ON incubation under standard conditions to ensure firm attachment to the plate, the culture medium was removed, and 100 µL of resazurin WS was added to each well. Triplicate Blank samples were also prepared. Following 6 h of incubation, metabolized resazurin WS was transferred to a black 96-well plate for FI detection using the Spark multimode microplate reader (λ_{Ex}: 535 nm, λ_{Em}: 590 nm). Regression analysis of the curve's slope and *y*-intercept standard deviation (SD) [12, 13] yielded an LoB of ~18 cells/cm², an LoD of ~125 cells/cm², and an LoQ of ~400 cells/cm² (Fig. 2A).

To validate the estimated LoB, LoD and LoQ, 12 replicates of Blank and samples near LoD and LoQ limits were analyzed.

Experimental validation showed a relative repeatability of 54% for LoD and 21% for LoQ. Although the LoD signal significantly differed from the Blank ($P \leq 0.0001$, Cohen's d = 4.54), its low repeatability rendered it unreliable. Therefore, 400 cells/cm² (LoQ vs. Blank, $P \leq 0.0001$, Cohen's d = 14.94) was established as the minimum recommended cell concentration for reliable viability tests using the resazurin assay on the P-MSC/TERT308 cell line (Fig. 2B).

Repeatability, reproducibility, and measurement uncertainty

P-MSC/TERT308 cells were detached and counted as previously described, and 3 cell confluences were selected: $\sim 3 \times 10^4$ cells/cm² (high), 1.5×10^4 cells/cm² (medium), and 5×10^3 cells/cm² (low). Cells were diluted in the complete medium and seeded in quadruplicate

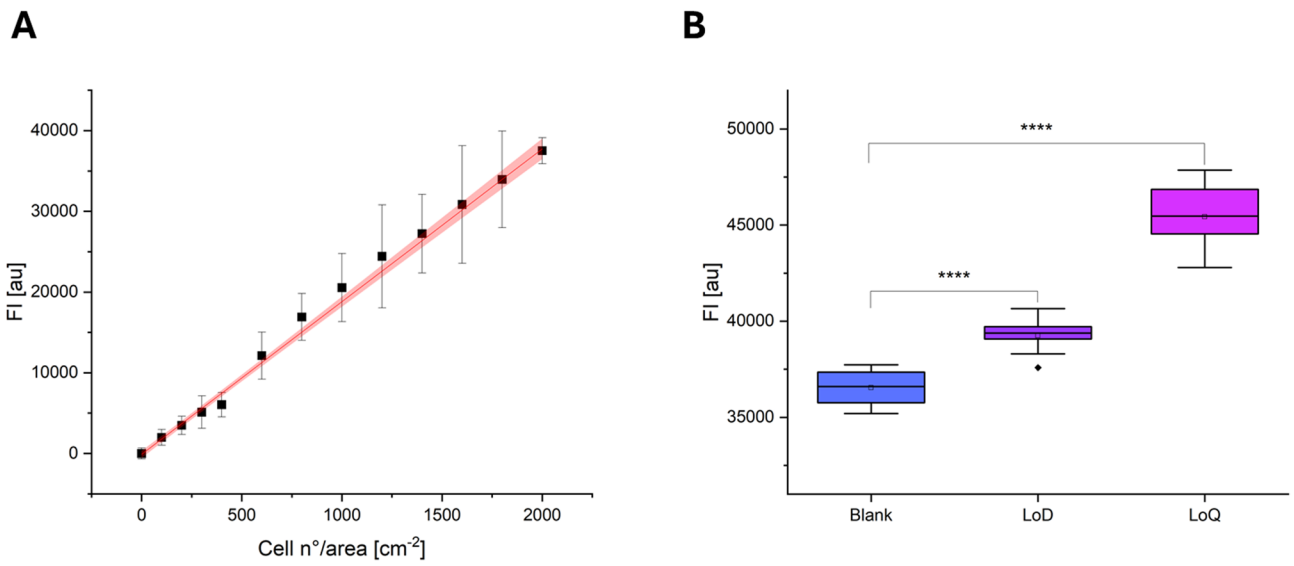

Fig. 2 **(A)** Estimation of Limits of Blank (LoB), Limits of Detection (LoD), and Limits of Quantification (LoQ) using the calibration curve method. $FI_{Sample-Blank}$ is plotted on the *y*-axis against cell concentration on the *x*-axis, with FI expressed in arbitrary units (au). Error bars represent SD. **(B)** Experimental validation of LoB, LoD and LoQ, with FI expressed in arbitrary units (au) on *y*-axis

into a pre-coated 96-well plate. After ON incubation under standard conditions to ensure firm attachment to the plate, the culture medium was removed, and 100 µL of resazurin WS was added to each well. Quadruplicate Blank samples were also prepared. Following 4 h of incubation under standard conditions, metabolized resazurin WS was transferred to a black 96-well plate for FI detection using the Spark multimode microplate reader (λ_{Ex}: 535 nm, λ_{Em}: 590 nm). This procedure was repeated 3 times (Exp1, Exp2, Exp 3) on different days by the same operator to assess repeatability, reproducibility, and measurement uncertainty (MU) [15, 16].

The results revealed a mean relative repeatability of 2.2% (1.3% for low, 3.2% for medium, and 2.1% for high confluency) and a mean relative reproducibility of 4.3% (1.7% for low, 8.1% for medium, and 3.2% for high confluency) (Fig. 3). With a 100 µL reading volume, the uncertainty attributed to pipetting error was 0.5% [17]. Previous instrumental repeatability experiments [15, 16] demonstrated a relative uncertainty below 0.5%, ensuring that instrumental fluctuations have minimal impact on the assay results. The mean relative expanded uncertainty (*U*%) for the resazurin assay was optimal at 9.8%, with values of 4.3% for low, 17.5% for medium, and 7.5% for high confluency. An MU of up to 20% is generally considered acceptable in toxicological analyses [18]. Therefore, the MU values observed in our study fall within the acceptable range.

Statistical analysis

FI data were expressed as the mean ± SD. Statistical analyses were conducted using Origin 2022 software (OriginLab Corporation).

To assess significant differences in FI values between sample groups, we performed a one-way analysis of variance (ANOVA) with post-hoc Tukey multiple comparisons. Statistical significance levels were denoted as follows: $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, and $^{****}P \le 0.0001$. All analyses with a P-value less than 0.05 were considered statistically significant.

Future directions

With the growing role of machine learning and computational modeling in scientific research, there is a significant potential to apply these technologies to predict optimal assay conditions, based on cell morphology, confluency, and other experimental variables. Integrating these tools into assay development could improve reliability and reproducibility.

Conclusions

Viability tests play a pivotal role in both basic and translational research, especially in cell-based pharmacogenomics for preclinical drug screening. Unfortunately, these studies often encounter challenges related to low inter-laboratory reproducibility, resulting in unreliable outcomes [3, 19, 20]. Over recent years, a worrying increase in the number of irreproducible preclinical studies has been observed, with laboratory protocols identified as the primary cause in 10.8% of cases [4]. Enhancing the reliability of results stands as a significant challenge in life sciences. Irrespective of the method employed to assess

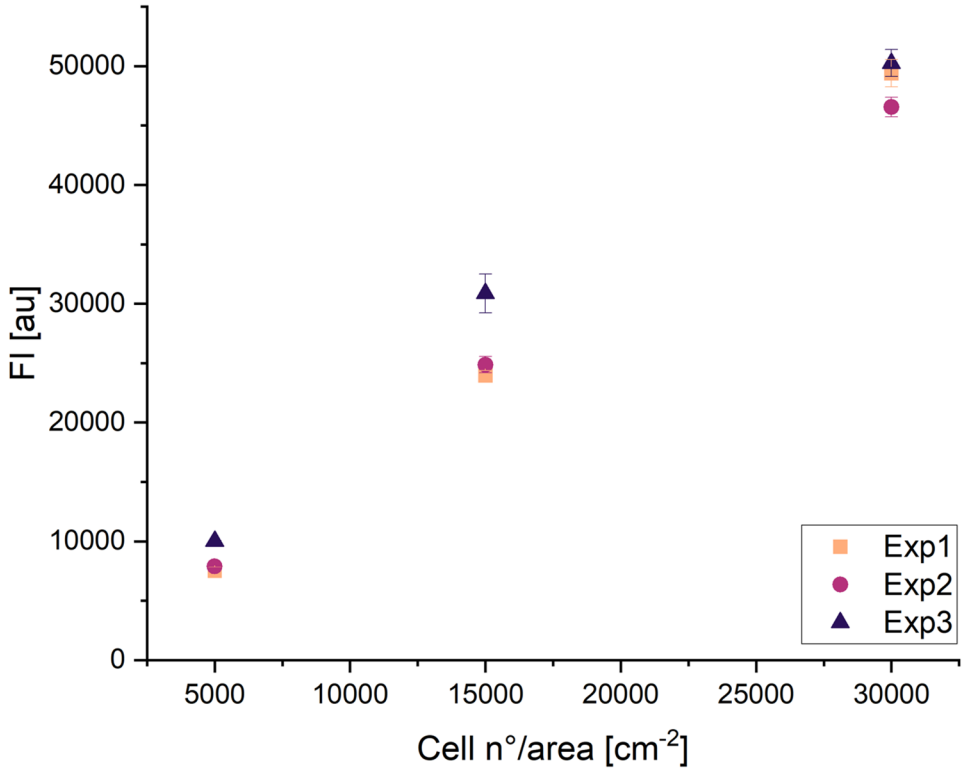

Fig. 3 Comparison of fluorescence intensity (FI) results (*y*-axis) obtained for each confluency condition (*x*-axis) in each experiment (Exp). FI is expressed in arbitrary units (au) and error bars represent standard deviation (SD)

cellular responses to treatment, the obtained results should exhibit consistency.

To face this challenge, a key strategy involves the optimization of experimental parameters, particularly laboratory protocols. In this work, we present the outcomes of a comprehensive optimization approach aimed at improving the reliability of the resazurin viability assay on the P-MSC/TERT308 cell line, a placenta-derived mesenchymal stem cell used within the LifeSaver project [2]. By applying our previously described and validated SOP for resazurin viability assay optimization [9, 10], we successfully fine-tuned the parameters for resazurin tests conducted on the P-MSC/TERT308 cell line and provided a new SOP (Additional file 1) tailored for these cells. The application of these SOPs consistently yielded reliable results, with a minimal MU of less than 10%.

Limitations

While this work advances the optimization of resazurin-based viability assays for placenta-derived mesenchymal stem cell lines, certain limitations should be noted. First, the specificity of the study to the P-MSC/TERT308 cell line may limit the generalizability of the findings to other cell types or experimental contexts. Second, despite efforts to minimize variability, intrinsic factors such as operator handling, cell characteristics (including passage number and viability), and the accuracy and precision of instrumentation can introduce variability, potentially affecting reproducibility across different laboratories. These factors underscore the need for broader validation across different cell lines and experimental conditions to ensure the robustness and applicability of the optimized protocol.

Abbreviations

FI	Fluorescence Intensity
LoB	Limit of Blank
LoD	Limit of Detection
LoQ	Limit of Quantification
ON	Overnight
SD	Standard deviation
WS	Working Solution
λ_{Em}	Emission wavelength
λ_{Ex}	Excitation wavelength

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-025-07298-w.

Supplementary Material 1: Title of data: INRIM_SOP Bio_003. Description of data: Step-by-step protocol to assay viability on P-MSC/TERT308 cell line in 2D culture using a resazurin-based method with optimized parameters to ensure consistent results with a measurement uncertainty < 10%

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Author contributions
Conceptualization, JP and CD; methodology, JP and LR; resources, CD; writing—original draft preparation, JP, LR and CD; supervision, CD; funding acquisition, CD.

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Data availability
All data generated or analyzed during this study are included in this published article and its supplementary information file.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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