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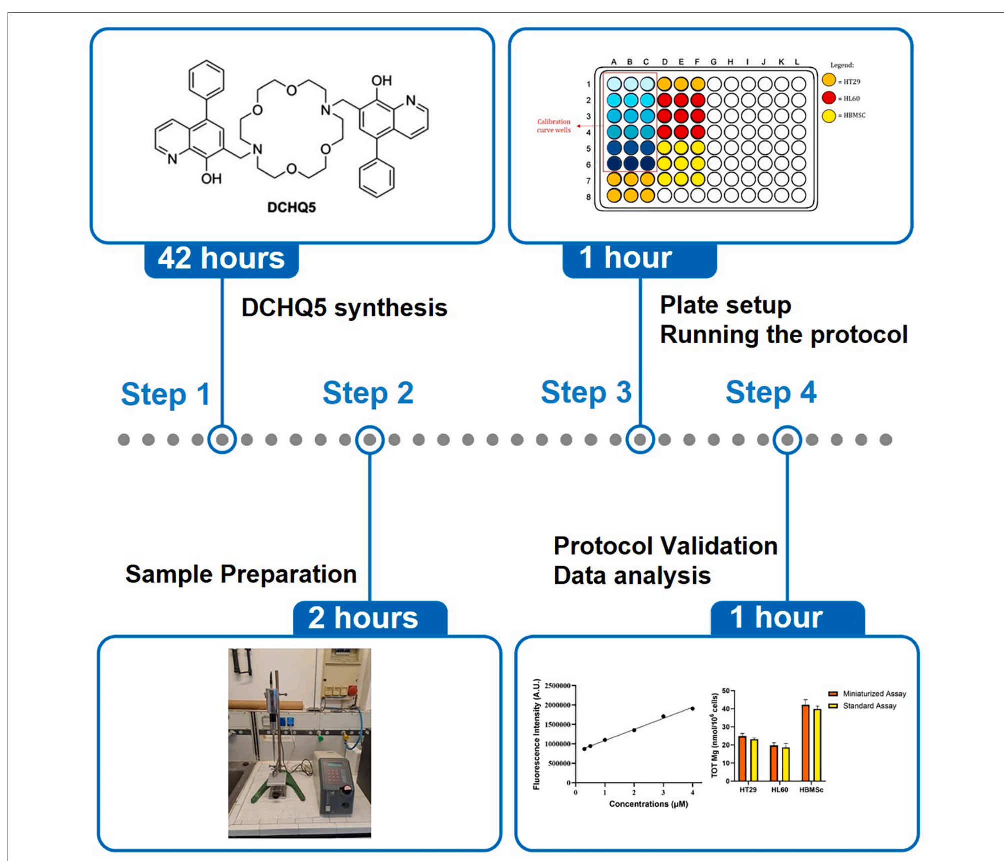
Protocol to quantify total intracellular magnesium in small samples using a fluorescent plate reader and fluorescent dye



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Highlights

Instruction for the synthesis of diaza-18-crown-6-hydroxyquinoline-5 (DCHQ5)

Steps for cell cultures and sample preparation

Details for $MgSO_4$ calibration curve building

Procedure for sample analysis and validation on cell samples using different cell lines

Assessing total magnesium (Mg) becomes challenging when cell quantities are minimal. Here, we present a protocol to quantify total intracellular Mg in samples with as few as several thousand cells using a fluorescent plate reader and the fluorescent dye diaza-18-crown-6-hydroxyquinoline-5 (DCHQ5). We describe steps for reagent setup, sample preparation, and assay running. We then detail procedures for sample analysis. This protocol has been validated on different cell lines with data from the literature.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Anconelli et al., STAR Protocols 6, 103956
September 19, 2025 © 2025 The Authors. Published by Elsevier Inc.
https://doi.org/10.1016/j.xpro.2025.103956

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Protocol

Protocol to quantify total intracellular magnesium in small samples using a fluorescent plate reader and fluorescent dye

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https://doi.org/10.1016/j.xpro.2025.103956

SUMMARY

Assessing total magnesium (Mg) becomes challenging when cell quantities are minimal. Here, we present a protocol to quantify total intracellular Mg in samples with as few as several thousand cells using a fluorescent plate reader and the fluorescent dye diaza-18-crown-6-hydroxyquinoline-5 (DCHQ5). We describe steps for reagent setup, sample preparation, and assay running. We then detail procedures for sample analysis. This protocol has been validated on different cell lines with data from the literature.

BEFORE YOU BEGIN

Despite its critical role in numerous biochemical processes,[1,2] magnesium (Mg) homeostasis remains incompletely understood. Flame Atomic Absorption Spectroscopy (F-AAS) is the primary standardized method for assessing total Mg; however, it requires large quantities of tissue or cell samples, which are not always readily available for every experiment. Alternative techniques, which utilize specific fluorescent dyes, typically measure free ionized magnesium (Mg^{++}). The fluorescent probe DCHQ5 (Figure 1), a dye based on diaza-18-crown-6-hydroxyquinoline derivatized by a phenyl group on each hydroxyquinoline arm, can bind both free and bound Mg, allowing for a simpler and more affordable fluorimetric assay. This method requires only 50,000–80,000 cells, as reported in previously published works.[3–6] Further reduction in sample size is necessary to broaden its applicability in routine laboratory settings.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
3-(N-morpholino)propanesulfonic acid (MOPS)	Sigma-Aldrich	Cat#M1254
Methanol (MeOH)	Sigma-Aldrich	Cat#34860
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#276855

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium chloride (NaCl)	Sigma-Aldrich	Cat#S9888
Potassium chloride (KCl)	Sigma-Aldrich	Cat#P3911
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma-Aldrich	Cat#P5379
Sodium hydrogen phosphate	Sigma-Aldrich	Cat# S9763
Magnesium sulfate	Sigma-Aldrich	Cat#M7506
Anhydrous calcium chloride	Sigma-Aldrich	Cat#C5670
5-Chloro-8-hydroxyquinoline	Sigma-Aldrich	Cat#C47000
p-Toluenesulfonyl chloride	Sigma-Aldrich	Cat#T35955
Sodium hydroxide	Sigma-Aldrich	Cat#221465
Acetone	Sigma-Aldrich	Cat#270725
Toluene	Sigma-Aldrich	Cat#34866
Phenylboronic acid	Sigma-Aldrich	Cat#78181
Anhydrous N, N-dimethylformamide	Sigma-Aldrich	Cat#227056
Tetrakis(triphenylphosphine)palladium(O)	Sigma-Aldrich	Cat#21666
Potassium carbonate	Sigma-Aldrich	Cat#60110
Ethanol	Sigma-Aldrich	Cat#459844
Anhydrous toluene	Sigma-Aldrich	Cat#244511
1,4,10,13-Tetraoxa-7,16-diazacyclooctadecane	Sigma-Aldrich	Cat#295809
Chloroform	Sigma-Aldrich	Cat#528730
Paraformaldehyde	Sigma-Aldrich	Cat#158127
Diethyl ether	Sigma-Aldrich	Cat#344362
Cyclohexane	Sigma-Aldrich	Cat#579191
Ethyl acetate	Sigma-Aldrich	Cat#270989
Silica gel for flash chromatography	Merck	Silicagel 60,230-430 mesh
Dulbecco's modified Eagle's medium (DMEM)	Dominique Dutscher	Cat#L00064-500
Rosewell Park medium 1640 (RPMI 1640)	Dominique Dutscher	Cat#L0501-500
Fetal bovine serum (FBS)	Gibco	Cat#10207-106
Glutamine	Euroclone	Cat#3000D
Trypsin/EDTA	Sigma-Aldrich	Cat#T4174
Experimental models: Human cells		
HT29	ATCC	Cat#HTB-38
HL60	ATCC	Cat#CCL-240
BMSc	CGT Global	Cat#BMMSC001C
Saos-2	ATCC	Cat#HTB-85
Software and algorithms		
GraphPad Prism	DotMatics	Prism - GraphPad

MATERIALS AND EQUIPMENT

Phosphate Buffer Saline (PBS)

Chemical	Final concentration	Amount
NaCl	137 mM	8.00 g
KCl	2.7 mM	0.20 g
KH_2PO_4	1.5 mM	0.20 g
Na_2HPO_4	8.1 mM	1.15 g
Milli-Q Water	N/A	up to 1 L

For PBS solution

Timing: 1 h

- Put all the powder in a 1 L volumetric flask and add Milli-Q Water to reach the final volume.
- Sterilize by filtering with sterile filters with 0.2 μ mesh or by autoclaving.

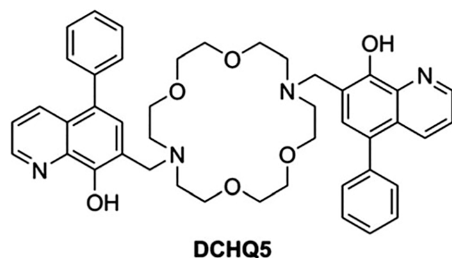

Figure 1. DCHQ5 dye structure

- This solution can be stored at 20°C for several months

⚠ CRITICAL: It is important to use PBS without Ca^{++} and Mg^{++} to avoid the presence of exogenous magnesium. To store, avoid glass bottles and flasks. Use, instead, good plastic vessels to avoid Mg release.

Buffer 2 mM MOPS:MeOH 1:1 (pH 7.4)		
Chemical	Final concentration	Amount
MOPS 4 mM	2 mM	50 mL
MeOH 99.9%	50% v/v	50 mL

First phase: 4 mM MOPS solution

⏲ Timing: 30 min

- To prepare 100 mL of 4 mM MOPS, weigh 83.86 mg of MOPS and dissolve them in ultrapure (or Milli-Q) water. Check pH and set it up at 7.4.
- This solution can be stored at +4°C for several months.

⚠ CRITICAL: To store, avoid glass bottles and flasks. Use, instead, good plastic ones to avoid Mg release.

Second phase: Final buffer

⏲ Timing: 5 min

- To obtain 100 mL of 2 mM MOPS:MeOH pH 7.4 add 50 mL of 4 mM MOPS solution and 50 mL of 99.9% MeOH.
- This solution can be stored at +4°C for several months in a tightly closed bottle.

⚠ CRITICAL: To store, avoid glass bottles and flasks. Use, instead, good plastic ones to avoid Mg release.

For DCHQ5 dye solutions

⏲ Timing: 30 min

DCHQ5 synthesis has been performed according to Sargenti et al. 2017[3] and it's reported in the "step-by-step method details."

- To prepare the Starting Solution of DCHQ5, dissolve 1 mg of DCHQ5 powder in 1 mL of DMSO to obtain a concentration 1.37 mM.

Note: This solution can be aliquoted and stored at 4°C for a year.

⚠ CRITICAL: To ensure the complete solubilization sonicate for 10 min in the Ultrasonic bath. Avoid repeated freeze and thawing.

- Working Solution preparation: add 12.2 μL of the DCHQ5 Starting Solution and 987.8 μL of MOPS: MeOH pH 7.4 to prepare 1 mL of the working solution 0.0167 mM, resuspending accurately.

⚠ CRITICAL: This solution must be prepared just before the beginning of the essay.

For $MgSO_4$ stock and subsequent dilution

Timing: 30 min

- Prepare 1 mM stock solution of $MgSO_4$ in Milli-Q water.
- The diluted solutions (3, 5, 10, 20, 30, 40) are prepared following this scheme:

$MgSO_4$ concentration	$MgSO_4$ stock solution	MOPS:MeOH	Final volume
3 μM	3 μL	997 μL	1,000 μL
5 μM	5 μL	995 μL	1,000 μL
10 μM	10 μL	990 μL	1,000 μL
20 μM	20 μL	980 μL	1,000 μL
30 μM	30 μL	970 μL	1,000 μL
40 μM	40 μL	960 μL	1,000 μL

Note: These solutions can be stored at +4°C for several months.

Equipment notes

Sonicator: Ultrasonic Processor XL – Misonix, equipped with a microtip (1.6 mm diameter) to operate on volume between 0.2 and 5 mL (Figure 2). Thanks to interchangeable tips and adjustable power settings, this sonicator allows efficient disruption of cellular suspension, ensuring the release of Magnesium (Mg) from all intracellular compartments where it is bound.

Alternatives: Other sonicator with different tips may be used. Attention should be paid to the size of the tip. The sonicator was set to deliver a constant power of 100 W, with a minimum sonication time of 20 s per sample. Performing the procedure on ice is not necessary.

Plate reader: PerkinElmer Ensight. This multimodal reader is capable of measuring absorbance, fluorescence, and luminescence (Figure 3). The device operates with Kaleido v. 3.0.3067.117 software, which enables easy and rapid export of measurement in CSV format. These files can be analyzed using software such as Excel or GraphPad Prism.

Alternatives: Any microplate reader capable of fluorescence detection (e.g., Molecular Devices, Promega, Thermo Scientific) could be used.

To perform the assay, a specific protocol is needed. Its details are shown in the Figure 4.

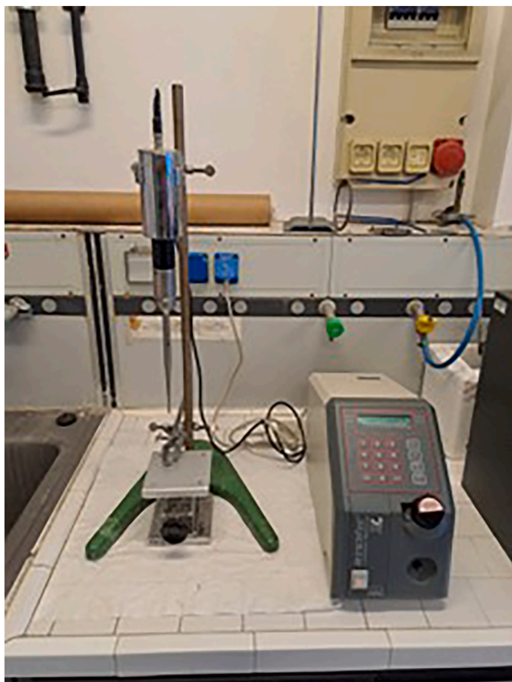

Figure 2. Ultrasonic Processor XL – Misonix

The excitation and emission wavelengths have been optimized for our instrument and working conditions. We recommend that users optimize these settings according to their own equipment and experimental parameters prior to running the assay. The height has been optimized based on the plate type used in our set up. This parameter may need to be adjusted depending on the conditions employed. To ensure the proper mixing inside each well, a shaking phase was included before the reading.

STEP-BY-STEP METHOD DETAILS

DCHQ5 synthesis

Timing: 42 h

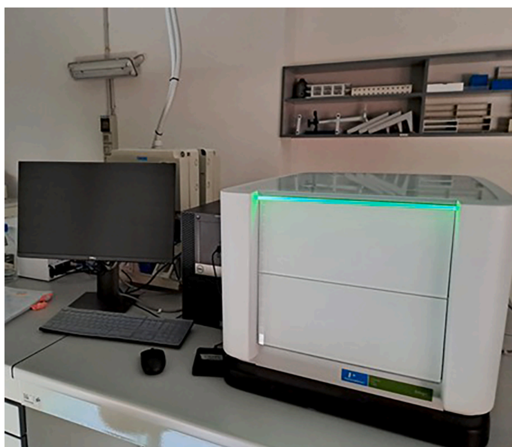

Figure 3. PerkinElmer Ensight plate reader

Measurement Sequence > Shake 1

Shake Mode: Linear
Duration: 00:00:10.000 hh:mm:ss.sss
Speed: 400 rpm
Diameter: 3 mm
Plate Location: Outside

Measurement Sequence > Fluorescence intensity 3

Measurement Mode: Single
Measure from: Top
Excitation Wavelength: 362 nm
Emission Wavelength: 510 nm
Measurement Height: 8 nm
Number of Flashes: 1000
Optimization

Figure 4. Kaleido Protocol for the miniaturized assay

Timing: 7–8 h (for step 1)

Timing: 8–10 h (for step 2)

Timing: 3–6 h (for step 3)

Timing: 20–22 h (for step 4)

The synthesis of the fluorescent dye DCHQ5 was performed according to the microwave as described in detail by Sargenti et al.[3] A summary of the procedure is provided below, and a scheme is reported in Figure 5.

1. Synthesis of 5-chloro-8-tosyloxyquinoline (1).
   a. Prepare a 50-ml one-neck round-bottom flask with a Teflon-coated magnetic stir bar.
   b. Add 21 ml of 1 M sodium hydroxide aqueous solution (21 mmol) to the round-bottomed flask.
   c. Dissolve 3.59 g of 5-chloro-8-hydroxyquinoline (20 mmol) by adding it to the aqueous solution.
   d. Dissolve 3.81 g of p-toluenesulfonyl chloride (20 mmol) in 8 ml of acetone.
   e. Add the solution of p-toluenesulfonyl chloride the reaction mixture drop by drop over 5 min with stirring.
   f. Vigorously stir the heterogeneous reaction mixture on a magnetic stirrer at 20°C for 5 h.
   g. Add 20 ml of distilled water to the reaction mixture.
   h. Filter the solid using a fritted filter funnel under vacuum and wash the solid twice with 10 ml of water.
   i. Recrystallize the crude product from toluene to obtain 6.07 g of 5-chloro-8-tosyloxyquinoline (18.2 mmol, 91% yield).
2. Synthesis of 5-phenyl-8-tosyloxyquinoline (2).

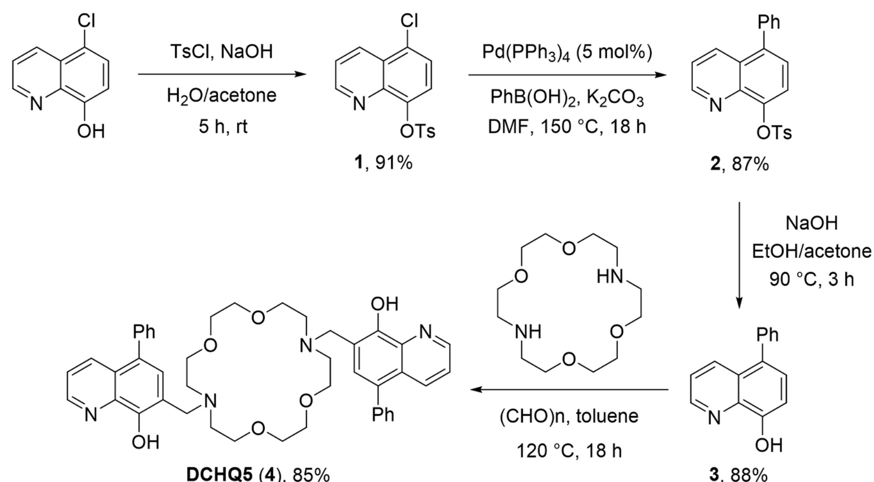

Figure 5. DCHQ5 synthesis

a. Prepare a 25-ml three-neck round-bottomed flask, a reflux condenser with an inlet glass vacuum hose adapter and a Teflon-coated magnetic stir bar.
b. Connect the apparatus to a single-manifold Schlenk line attached to an inert gas supply (nitrogen or argon) using the inlet hose adapter.
c. Flame-dry the glassware and allow it to cool down under vacuum before refilling it with inert gas.
d. Add 0.668 g of 5-chloro-8-tosyloxyquinoline (2 mmol) to the round-bottomed flask.
e. Add 10 ml of anhydrous N,N-dimethylformamide.
f. Add 0.116 g of tetrakis(triphenylphosphine)palladium(0) (0.1 mmol, 5 mol%) to the reaction mixture and allow it to stir for 10 min at 20°C temperature.
g. Add 0.256 g of phenylboronic acid (2.10 mmol), followed by 0.58 g of K_2CO_3 (4.2 mmol).
h. Place the flask in a pre-heated oil bath (150°C) on a hot-plate magnetic stirrer.
i. Stir the reaction mixture at reflux for 5 h under nitrogen atmosphere.
j. Cool the reaction mixture to 20°C temperature and add 10 ml of dichloromethane.
k. Wash the organic layer twice with 10 ml of distilled water each time and twice with 5 ml of a 5% aqueous lithium chloride solution each time.
l. Dry the organic layer over anhydrous Na_2SO_4 (2 g) and filter the mixture through a fritted filter funnel.
m. Remove the solvent using a rotary evaporator (bath temperature: 30°C).
n. Prepare a chromatography column (diameter: 2 cm; height: 15 cm) packed with silica gel by using cyclohexane:ethyl acetate 8:2 (vol/vol).
o. Dissolve the crude product in ~1 ml of the eluent and drip the resulting solution onto the top of the silica gel column.
p. Perform the purification by eluting with an 8:2 (vol/vol) mixture of cyclohexane and ethyl acetate. Collect the fractions containing the product and remove the solvent using a rotary evaporator (bath temperature: 30°C) to obtain 0.65 g of Synthesis of 5-phenyl-8-tosyloxyquinoline as a faint yellow solid (1.73 mmol, 87% yield).

3. Synthesis of 5-phenyl-8-hydroxyquinoline (3).
   a. Prepare a 25-ml two-neck round-bottomed flask with a reflux condenser and a Teflon-coated magnetic stir bar.
   b. Add 10 ml of ethanol: acetone 1:1 (vol/vol) to the round-bottomed flask.
   c. Add 0.751 g of 5-phenyl-8-tosyloxyquinoline (2 mmol) to the round-bottomed flask with stirring.
   d. Add 6 mL of 1 M sodium hydroxide aqueous solution (6 mmol) to the round-bottomed flask with stirring.

e. Put the flask in a pre-heated oil bath (90°C) on a hot-plate magnetic stirrer.
f. Stir the reaction mixture at reflux for 3 h under air.
g. Cool the reaction mixture to 20°C temperature and add 10 ml of distilled water.
h. Adjust the pH of the solution to ~6.5 by adding a 1 M hydrochloric acid aqueous solution drop by drop.
i. Filter the remaining solid using a fritted filter funnel under vacuum and wash it twice with 10 ml of distilled water.
j. Alternatively, instead of filtering, extract the product from the aqueous layer using dichloromethane (3 extractions with 10 ml of organic solvent each time).
k. Recrystallize the crude product using methanol to obtain 0.39 g of 5-phenyl-8-hydroxyquinoline 3 (1.76 mmol, 88% yield) as a yellow solid.

4. Synthesis of DCHQ5 (4).
Prepare a 10-ml two-neck round-bottomed flask with a reflux condenser, an inlet glass vacuum hose adapter and a Teflon-coated magnetic stir bar.
a. Connect the apparatus to a single-manifold Schlenk line attached to a nitrogen gas supply using the inlet hose adapter.
b. Flame-dry the glassware and allow it to cool down under vacuum before refilling it with nitrogen.
c. Add 0.066 g of 5-phenyl-8-hydroxyquinoline (0.3 mmol) to the round-bottomed flask.
d. Add 2 ml of anhydrous toluene.
e. Add 0.039 g of 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (0.15 mmol) with stirring.
f. Add 0.009 g of paraformaldehyde (0.3 mmol) with stirring.
g. Place the flask in a pre-heated oil bath (120°C) on a hot-plate magnetic stirrer.
h. Stir the reaction mixture at reflux for 18 h under nitrogen atmosphere.
i. Cool the reaction mixture to 20°C temperature and remove the organic solvent using a rotary evaporator (bath temperature: 50°C) to obtain a yellow powder.
j. Check the reaction conversion by 1H-NMR spectroscopy of the crude product.
k. Recrystallize the crude product by dissolving it in hot chloroform (0.5 ml) and by adding cold methanol (2 ml) to obtain 0.093 g of pure DCHQ5 (4) as a yellow powder (0.128 mmol, 85% yield).

⚠ CRITICAL: The DCHQ5 powder can be stored at 20°C temperature for several months without any appreciable decomposition.

Adherent cell culture

Timing: 30 min

The human osteosarcoma cell line Saos-2, human colon adenocarcinoma cell line HT29 and human bone marrow-derived mesenchymal stem cells HBMSC were grown in complete medium obtained by supplementing the basal medium with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The basal medium used for Saos-2 was RPMI-1640, for HT29 and HBMSC was Dulbecco's Modified Eagle's Medium (DMEM). All the cultures were maintained at 37°C in a humidified incubator with 5% of CO2. Adherent cells (Saos-2, HT29 and HBMSC) were seeded at a density of 10.000 cell/cm^2 in T25 flasks. Culture medium was replaced every 3 days. The cells were divided when they reached 80% confluence.

5. To detach cells, discard the medium and wash the culture surface twice with 5 mL of PBS.
6. Add 1 mL of trypsin /EDTA solution to cover all the surface and place in the incubator at 37°C for 10 min.
7. Put the flask under the inverted microscope and observe if the cells are detached. If not, allow them to remain in the incubator for 5 min more.

Table 1. Minimum number of cells to run the assay	
Experimental model: Cell lines	N° of cells needed
HT29	1,300
HL60	1,800
HBMSc	1,000
Saos-2	1,400

8. Add 4 mL of complete medium to block the proteolytic activity of trypsin.
9. Suspend well to have a monodispersed cell suspension and count them.
10. Seed the cells at a density of 10.000 cells/cm^2 in T25 with a final volume of complete medium of 5 mL.
11. When the cells have to remain in culture, replace the exhausted medium with the fresh one every 3-4 days.

Suspended cell culture

Timing: 20 min

Human promyelocytic leukemia cell line HL60 were grown in complete medium obtained by supplementing the RPMI-1640 with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The cultures were maintained at 37°C in a humidified incubator with 5% of CO_2.

12. Suspend well the cultured cells and count them.
13. Seed the cell in a T25 flask at a density of 100.000 cells/mL in a final volume of 5 mL.
14. When the cells have to remain in culture, replace the exhausted medium with the fresh one every 3-4 days.
15. Subculture every 3 days by a 1:3 dilution with fresh medium.

Cell preparation

Timing: 1 h

This step outlines the procedure for preparing cell samples for the assay.

Note: This protocol is suitable for all cell types. It is important to note that the minimum number of analyzable cells depends on their Mg content. Since the limit of detection for this method is 0.1 µM of Mg, the sample must contain a sufficient amount of magnesium to reach this threshold (the minimum number is reported in "expected outcomes and Validation" point 2, Table 1).

16. Harvesting and washing.
    a. Once cells are ready for the experiment, detach them with 0.25% Trypsin/1 mM EDTA.
    b. Count them twice.
    c. Wash twice with calcium and magnesium-free PBS pH 7.2, centrifuging at 250g × 10 min.
    d. Before the second centrifugation, count the cells again at least twice, pellet the cells and resuspend them in 200 µL of 4 mM MOPS pH 7.4 if the total cell number is between 5000 and 150000. If the cell number exceeds 150000, resuspend the pellet in 2 mM MOPS:MeOH pH 7.4 to obtain a final concentration of 10^6 cells/mL.

⚠ CRITICAL: Use only PBS without calcium and magnesium to prevent contamination with trace magnesium from the buffer. Accurate cell counting is essential for consistent and reproducible results, do not underestimate this step.

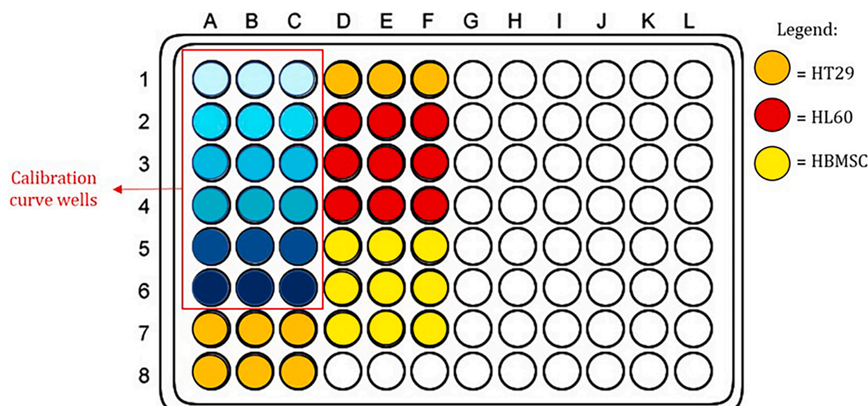

Figure 6. A schematic representation of the plate used for the first validation experiment
On the left, in a blue scale, different solutions of $MgSO_4$ are shown (1A-1C 3 μM, 2A-2C 5 μM, 3A-3C 10 μM, 4A-4C 20 μM, 5A-5C 30 μM, 6A-6C 40 μM).

17. Sample disruption.
    a. To ensure the total disruption of all the cellular components, sonicate each sample for at least 20 s at 100 W.

⚠ CRITICAL: Incomplete sonication may result in inaccurate intracellular magnesium quantification.

Note: For optimal assay performance, use a black 96-well plate non-binding with opaque bottomed.

Calibration curve

⏲ Timing: 1 h

18. Turn on the fluorescent plate reader and load the Ensight measurement sequence as mentioned above.

⚠ CRITICAL: Warm up the instrument to prevent macroscopic errors in fluorescence reading.

19. Add 180 μL of 15 μM DCHQ5 Working Solution and 20 μL of specific $MgSO_4$ solution in each well.

Note: The final concentration of DCHQ5 solution per well is 13.5 μM. Prepare at least 3 wells for each concentration, as showed in Figure 6.

Sample analysis

⏲ Timing: 1 h

The preparation of samples differs depending on the number of cells in the samples, keeping in mind that the amount of Magnesium has to be over the LOD.

20. If the cells are more than 150.000, suspend them in 2 mM MOPS:MEOH pH 7.4 to achieve a final concentration of 10^6 cells/mL.

21. For each well, add 180 μL of 15 μM DCHQ5 Working Solution in 2 mM MOPS:MeOH pH 7.4, then 20 μL of the sonicated cell solution, which correspond to 20.000 cells. The final DCHQ5 concentration in the wells is 13.5 μM.
22. For 5000 to 150000 cells, suspend the cells in 200 μL of 4 mM MOPS that is the minimum volume to perform the assay in duplicate.
23. For each well, add the following in this order: 98.9 μL of sample, 98.9 μL of MEOH and 2.2 μL of 1.37 mM DCHQ5 Starting Solution, to obtain a DCHQ5 concentration 13.5 μM in the wells.
24. Resuspend accurately the samples for 3 times.

⚠ CRITICAL: Avoid delaying the reading step to prevent fluorescence quenching of the dye.

EXPECTED OUTCOMES

An important improvement of this method, compared to previously published protocol,[3] is the lowered Limit of Detection (LOD). The calculus of the LOD has been performed using the formula reported in the point 3 of "quantification and statistical analysis," while the data can be found in Data S1. Our final LOD is 0.1 μM, which is half of the previously reported LOD of 0.2 μM[3]. The improvement of the LOD is surely connected to the removing of PBS which is reported to have a quenching effect on the probe.

To assess the accuracy of the miniaturized DCHQ5 method, total Mg levels measured in various cell types using this assay were compared to those determined using the previously published method.[3–6] Mg concentrations have been quantified in HT29 colon adenocarcinoma cells, as a model of adherent cells, and in HL60 leukemic cells, representing a suspension culture model. We also examined primary bone marrow mesenchymal stem cells (HBMSc), isolated from a healthy donor through an invasive procedure which yields a limited number of cells. For all experiments, the assayed volume is corresponding to a sample of 5000 cells for each well. The plate disposition is reported in Figure 6. Each group displayed a characteristic fluorescence based on their level of Mg content.

The data analysis could be found in Data S1. From the comparison with already published data, has emerged that the new miniaturized protocol results are comparable with the already published ones[6,7] (Figure 7).

In the Table 1, it's reported the minimum number of cells needed to overcome the LOD:

A second validation was conducted by analyzing the well-known correlation between intracellular Mg levels and cell cycle progression in Saos-2 cells (5000 cells per experimental point), specifically examining the percentage of cells in the G_0/G_1 phase, as reported in Figures 8 and 9.[1,8] As mentioned earlier, the emitted fluorescence is proportional to the Mg content. After 24 h from seeding, the Mg concentration is approximately 13 nmol/10^6 cells. Over the next 72-96 h in culture, there is a significant increase in intracellular Mg, reaching up to 18 nmol/10^6 cells. Once confluence is reached after approximately 96 h of culture, cell cycle retardation occurs, and intracellular Mg decreases to around 10 nmol/10^6 cells. Figure 10 shows the inverse correlation between Mg levels and the percentage of cells in the G1 phase.

QUANTIFICATION AND STATISTICAL ANALYSIS

The Ensight plate-reader uses Kaleido software, which facilitates the efficient export of data into Excel. Statistical analysis should be performed using software such as Excel or GraphPad Prism to determine significant values between different conditions and the controls/references.

1. Calibration Curve building.

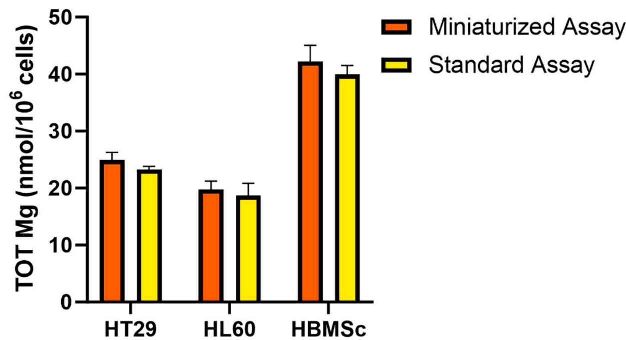

Figure 7. Total Mg content in different cell lines: comparison between the miniaturized assay and the standard assay

Data reported as mean ± SD, p = 0.05.

From the data derived from the reading it is possible to derive an average fluorescence and with each concentration a calibration curve is drawn. In Figure 10 an example with GraphPad is reported. The curve will be described from a typical equation of a straight line:

$$Fluorescence\ Intensity\ (F)\ =\ a + b[Mg]$$

2. Once the procedure is complete, quantification can be performed directly in the samples. First, it is important to determine the Mg concentration (μM) in the well from the average fluorescence (F) of three replicates:

$$[Mg]\ =\ \frac{F\ -\ a}{b}$$

3. The results for this type of measurement are reported as nanomoles of Mg per million cells. The last step is the conversion of the Mg concentration into nmol/10^6 cells. To do this, it is important to know the cell count per aliquot.

$$Total\ Mg\ content\ \left(\frac{nmol}{10^6 cells}\right)\ =\ \frac{[Mg]\ x\ V\ x\ 10^6}{n}$$

V=total volume per well.

n=number of cells per well.

4. LOD quantification.

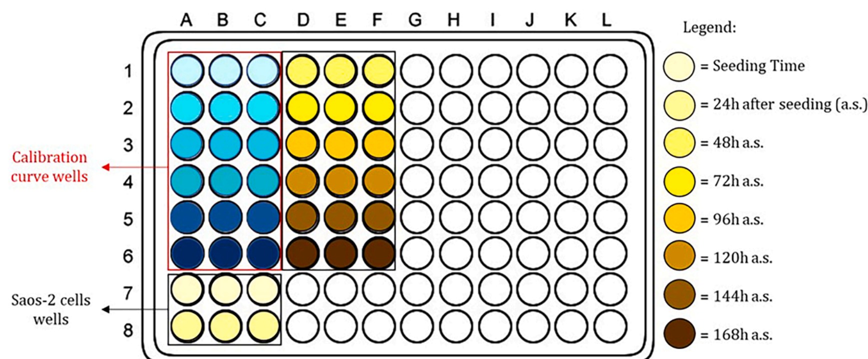

Figure 8. A schematic representation of the plate used for the second validation experiment

On the left, in a blue scale, different solutions of $MgSO_4$ are displayed (1A-1C 3 μM, 2A-2C 5 μM, 3A-3C 10 μM, 4A-4C 20 μM, 5A-5C 30 μM, 6A-6C 40 μM).

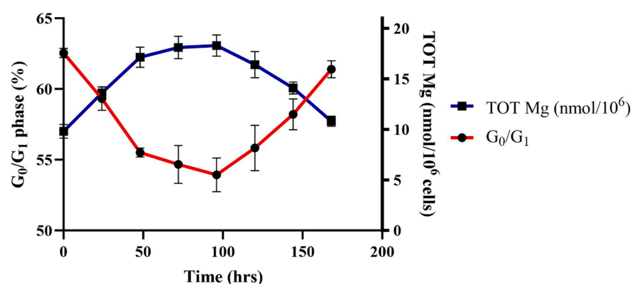

Figure 9. Graph showing the relationship between intracellular Mg content and the percentage of Saos-2 cells in the G0/G1 phase as a function of the time after seeding

Data reported as mean ± SD, $p = 0.05$.

The standard formula used to determine the Limit of Detection (LOD) for a linear calibration curve is the following one:

$$LOD = \frac{3.3 \ x \ SD}{a}$$

SD= Standard Deviation.

a= calibration curve interpolation value.

LIMITATIONS

This assay relies on accurate cell counting, so it is important to reserve a particular attention during the counting phase.

A major limitation of the assay lies in the health status of the cells, particularly the death rate within the population. If the percentage of dead cells is very high, these cells could release magnesium but still be counted, leading to misleading results and incorrect conclusions.

TROUBLESHOOTING

Independent trials give inconsistent results.

Problem 1

The samples are not properly prepared.

Potential solution

- The counting process was not correct and the sample is too diluted for the assay. See the section "cell preparation" in the "step-by-step method details."

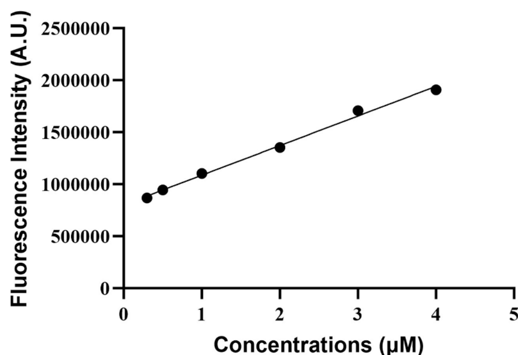

Figure 10. Typical calibration curve

- Ensure that the final cell concentration in the sample is below 10^6 cells/mL avoid exceeding the Critical Micellar Concentration. Refer to the "sample preparation" in the "step-by-step method details."
- The cells were not totally disrupted. If membranes or organelles remain intact, the probe may yield unreliable results. See the section "cell preparation" in the "step-by-step method details."

Problem 2

The fluorescence measurements of the technical replicates give inconsistent results.

Potential solution

- The dye was not properly resuspended. Mix the sample three more times before re-reading the entire plate. Check the "sample preparation" in the "step-by-step method details."
- The plate-reader is not correctly set-up. Refer to "Figure 3" in the "equipment notes."
- The instrument reading was inaccurate. Shake well and repeat the reading (max 2 times to avoid quenching effects). Check out the "equipment notes" in "materials and equipment."
- Magnesium is too low to be detected. Every cell line has its own Mg level, however could change dependently from the cell culture condition and treatment. We suggest to perform preliminary test to assess the range of variation.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Stefano Iotti (stefano.iotti@unibo.it).

Technical contact

The technical contact is Lorenzo Anconelli (lorenzo.anconelli3@unibo.it).

Materials availability

The dye DCHQ5 could be synthetized following the reported protocol or could be available upon request by contacting lead contact.

Data and code availability

Not applicable.

ACKNOWLEDGMENTS

This study was funded by the European Union NextGenerationEU, in the framework of the Biomineralization in senescent human mesenchymal stem cells – an in-depth multi-modal and multi-scale supra-molecular and sub-molecular study (XMINE) PRIN 2022, project n. 2022KM2MBT_002 CUP J53D23008710006. The views and opinions expressed are solely those of the authors and do not necessarily reflect those of the European Union, nor can the European Union be held responsible for them.

AUTHOR CONTRIBUTIONS

L.A., G.F., and M.M.R. developed and optimized the assay. F.R. performed the data analysis. M.L., C.C., M.R., J.A.M., and S.I. supervised the project and wrote the manuscript. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.103956.

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