MATERIALS AND METHODS

Reagents

HMGR, HMG-CoA, NADPH, NADP⁺, CoA, potassium phosphate, sodium phosphate, magnesium sulfate, phenyl-methanesulfonyl-fluoride, tosyl-phenyl-alanyl-chloromethyl-ketone, EDTA, DTT, and DMSO were all purchased from Sigma-Aldrich. HPLC grade methanol was obtained from JT Baker. All solvents and reagents were of the highest purity available.

HPLC analysis

A HPLC system Gold (Beckman Coulter Inc.) equipped with a UV/VIS detector and HPLC column heater (Alltech) was used for the analysis. Reaction mixture consisting of HMGR (0.4 µM), NADPH (2.68 mM), and HMG-CoA (1.55 µM) diluted in the activity buffer was incubated at 37°C, and aliquots were withdrawn at indicated times and separated by HPLC. Each species (both isolated analytes and incubation mixtures) were injected and separated on a reverse phase Phenomenex Luna C18 column (5µm particle size, 250 × 4.6 mm equipped with a 5 mm guard column of the same phase), and thermostated at 26°C, with the following linear gradient of potassium phosphate 100 mM (solvent A) and methanol (solvent B): 10%–30% B up to 15 min and 30%–10% B for 5 min at flow rate of 0.8 ml/min, UV/VIS detector set at 260 nm. An injection volume of 10 µl was used throughout. After each chromatographic elution, column was regenerated with two column volumes of 60% methanol. HMG-CoA, NADPH, and NADP⁺ were directly monitored, whereas mevalonate was determined by monitoring CoA production (mevalonate/CoA 1:1 stoichiometric ratio; Fig. 1). Enzyme catalytic activity was performed at 37°C in a 100 mM sodium phosphate buffer containing 1 mM EDTA, 10 mM DTT, 2% DMSO, and 1 mM magnesium sulfate, pH 6.8, following the reaction for different times of incubation. No interfering signal was detected upon injection of the activity buffer. Each analysis was repeated in quadruplicate. Detection

Fig. 1. Schematic representation of HMGR enzymatic reaction.
of the HMGR levels and activity in cell homogenate was carried out on human colon cancer cell line HCT116, previously shown to express HMGR (17). Cells were suspended in the activity buffer added with phenyl-methane-sulfonyl-fluoride and tosyl-phenyl-alanyl-chloromethyl-ketone as proteases inhibitors and lysated using an insulin syringe. Total cellular enzyme levels were quantified according to the standard addition method (18); different amounts of the isolated enzyme (30–130 ng per mg of total proteins) were added to the reaction mixture (NADPH, HMG-CoA, and cell lysate, dissolved in the activity buffer) and detecting the product of CoA after 60 min. This mixture was finally centrifuged at 10,000 g for 10 min. These quantities were

Fig. 2. Calibration curves of CoA, NADPH, HMG-CoA, and NADP⁺. Linear fits (solid lines) and 95% confidence bound (dotted lines) are reported. Each experimental point was the average of three replicates.

Fig. 3. A: Representative chromatographic profiles of reaction mixture in the absence (dashed line) and in the presence (after 60 min of incubation) of HMGR (solid line). B: Chromatographic profiles of cell lysate in the absence (gray line) and in the presence of reaction mixture after 0 and 60 min of incubation (dashed and solid line, respectively). Methanol gradient is reported (dotted line).
The same method was successfully applied to a cellular lysate, reporting comparable chromatographic profiles without any significant interfering compounds (Fig. 3B). Multiple standard additions of the isolated enzyme to reaction mixture containing cell lysate showed a linear correlation between CoA/mevalonate produced and total enzyme. The measured concentration of the cellular active enzyme was 52 ± 2 ng/mg of total proteins (Fig. 5), corresponding to 15.58 ± 0.48 units/mg cell protein, consistent with previously studies on tumor cells (9).

Finally, the proposed method was validated by comparison with a commercially available spectrophotometric assay kit (Cod. CS1090, Sigma-Aldrich), which provided comparable levels of HMGR in HCT116 cell lysates (14.8 ± 0.4 units/mg of total cell proteins).

In conclusion, this method could represent a useful tool for both rapid and low-cost routine assays of HMGR activity, and quantitation of all the species involved in the equilibrium.

### RESULTS AND DISCUSSION

Calibration curves were established for each single analyte over adequate ranges of concentrations (Fig. 2). Dynamic ranges of linearity for the individual samples (determined by simple linear regression analysis), along with limits of detection (LODs) and limits of quantification (LOQs) (determined as ratio between 3 and 5 times the RMSE of the linear fit and the slope of the calibration curve, respectively) are reported in Table 1. The linearity of the standard curves was evaluated with a Chi-square goodness-of-fit test ($P > 0.05$). The substrates and the products of the enzymatic reaction were clearly and completely separated from each other, as shown in Fig. 3A.

Time course of mevalonate/CoA production was analyzed according to a mono-exponential model (Fig. 4), and bi-exponential analysis did not significantly improve the quality of the fit, as assessed with F-Test (95% confidence).

The assay reproducibility was evaluated by comparison of intra- and inter-day variability over 5 days by performing four replicates each day, with maximum variation coefficient associated to peak area $CV_{intra} = 5.48\%$ and $CV_{inter} = 6.31\%$, and to retention time $CV_{intra} = 0.14\%$ and $CV_{inter} = 0.23\%$.

| Analyte Tested | LOD   | LOQ   |
|----------------|-------|-------|
| NADPH         | 2.77 nmol | 4.62 nmol |
| NADP$^+$      | 1.33 nmol | 2.22 nmol |
| HMG-CoA       | 2.67 pmol | 4.45 pmol |
| CoA           | 0.27 pmol | 0.45 pmol |

normalized to the total cell lysate proteins detected using Bradford assay (19) with BSA as standard.

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