Original Article

New Luminescence-Based Approach to Measurement of Luciferase Gene Expression Reporter Activity and Adenosine Triphosphate-Based Determination of Cell Viability

(cell viability / reporter gene assay / luciferase / adenosine triphosphate)

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Abstract. The assay employing firefly luciferase as the end-point reporter is one of the most popular gene reporter systems. However, the physiological conditions of cells may affect the reporter gene expression, which makes an assessment of cell viability desirable. Estimates of cell viability may be based on different principles. We tested for correlations between various cell viability assessments, including luminescent determination of adenosine triphosphate in whole-cell lysate, and the reporter luciferase activity in pluripotent embryonic and colon adenocarcinoma cells. Luciferase activity in cell lysate from both cell lines cultured under different conditions correlated with the amount of viable cells assessed by all of the methods employed. Importantly, it was also possible to carry out adenosine triphosphate determination in cell lysates prepared in the buffer originally designed for determining luciferase activity; it correlated significantly with adenosine triphosphate determination in cells lysed in the buffer originally designed for adenosine triphosphate determination. The results suggest that the assessment of live cells by determining adenosine triphosphate can be multiplexed with a luciferase reporter gene assay, which allows independent monitoring of both reporter expression and cell viability.

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Abbreviations: A – absorbance, ATP – adenosine triphosphate, DMEM – Dulbecco's modified Eagle's medium, RA – retinoic acid, RLU – relative light units, TNF-α – tumour necrosis factor α.

Introduction

Genetic reporter systems efficiently analyse the activity of particular gene promoters and thus the transcription control under these promoters. One of the most popular gene reporter systems, both in transient and stable transfection experiments, is a reporter assay employing as the end-point reporter firefly luciferase, with its internal control represented by another constitutively expressed reporter such as β-galactosidase or Renilla luciferase (so-called dual reporter systems) (Martin et al., 1996; Grentzmann et al., 1998).

However, when making correlations between experimental treatments and the expression of a reporter gene, other events associated with cell physiology (cell death, cell type-dependent sensitivity to the promoter of control reporter, etc.) may affect the reporter gene expression. Thus, a dual reporter system is universally used. However, it may also cause several potential hurdles. In performing a transient transfection, the selection of a proper promoter directing the internal control reporter gene could be a complicated task. Further, insertion of additional genetic material, co-reporter for internal normalization, into a genome may contribute to non-specific dysregulation of gene expression in particular cells (Rosenberg, 1997; Bryja et al., 2003; Wurtele et al., 2003).

On the other hand, there are various methods for estimating the number of viable cells such as direct counting of cells, determination of cellular mass by total protein in the cellular lysate, determination of the metabolic activity of cells using tetrazolium salts, and by determination of the intracellular content of adenosine triphosphate (ATP) (Crouch et al., 1993; Goodwin et al., 1995; Petty et al., 1995). Nevertheless, various methods reveal a different sensitivity to the number of viable cells; therefore, the selection of a particular method may significantly influence the final results. Further, determination of cellular viability by any of these methods is usually performed in parallel samples since determination of the luciferase enzymatic activity is not compatible with cell viability assays.

For these reasons, we compared different cell viability assessments and the reporter luciferase activity in stably and transiently transfected pluripotent embryonic carcinoma P19 cells, stably carrying a retinoic acid (RA)-sensitive reporter (pRAREβ2-TK-luc), and human colon adenocarcinoma HT-29 cells, stably carrying a tumour necrosis factor α (TNF-α)-sensitive reporter (pBIIX-LUC). Our results suggest that the most convenient method for combined determination of luciferase activity and the number of viable cells is luminometric determination of luciferase activity and luminometric determination of ATP levels in the same cell lysate.

Material and Methods

Cell cultures and cell transfection

Human colon adenocarcinoma HT-29 cells and pluripotent embryonic carcinoma cell line P19 were cultured as described previously (Pachernik et al., 2005, 2007). Cell numbers in suspension were determined by a Coulter Counter (model ZM, Coulter Electronic, Luton, UK). HT-29 cells were stably transfected by the luciferase reporter construct NF-κB-driven plasmid pBIIX-LUC kindly donated by Dr. Saksela (Saksela and Baltimore, 1993). P19 cells were transfected with RA-sensitive luciferase reporter pRAREβ2-TK-luc plasmid (kindly provided by Dr. Glass, University of California, San Diego, CA). The reporter plasmids were co-transfected with pSV2neo neomycin-resistance plasmid into cells using electroporation (Bio-Rad Laboratories, Inc., Hercules, CA) and neomycin (G418 sulphate, Alexis, San Diego, CA)-resistant clones were selected as described previously (Novak et al., 2007; Hyzd'alova et al., 2008). Transient transfection was performed similarly without selection of neomycin-resistant clones as described previously (Pachernik et al., 2005, 2007).

For the experiments, P19 cells (4, 6, 8, 10 and 20 × 10^3 cells per cm^2) and HT-29 cells (2, 6, 14 and 20 × 10^3 cells per cm^2) were seeded for 24 h. Further, P19 and HT-29 cells were treated overnight with RA (0.2 μM, Sigma-Aldrich, St. Louis, MO) and by TNF-α (25 ng/ml; Peprotech, London, UK), respectively. Cells were harvested for viability and for luciferase activity assays. In the case of transient transfection, P19 cells were transfected by pRAREβ2-TK-luc together with CMV-β-galactosidase coding vectors (Pachernik et al., 2005, 2007). Cell suspensions were divided, seeded for 24 h, treated overnight with RA, and harvested as described above.

Determination of luciferase and β-galactosidase activity

The luciferase activity was determined using Luciferase Reporter Gene Assay (Roche, Mannheim, Germany) and compared with Luciferase Assay System (Promega, Madison, WI). The homogenized cell lysate (50 μl) sample was mixed with luciferase assay substrate (50 μl) and the emitted light was recorded by microplate luminometer LM-01T (Immunotech, Prague, Czech Republic) for 120 s. The intensity of the luminescence in relative light units (RLU) was expressed as the integral of the obtained kinetic curves. The β-galactosidase activity was measured using o-nitrophenyl-β-D-galactopyranoside as described previously (Bryja et al., 2003).

Cell viability assays

Determination of total cell proteins

Cells were washed by cold PBS, lysed in 70 μl of SDS buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 % glycerol, 1 % SDS, 1 mM EDTA), boiled, and the protein concentration was determined using BCA protein assay (Pierce, Rockford, IL).

Determination of cell metabolic activity by WST-1

Standard culture medium was replaced by cell culture medium supplemented with water-soluble tetrazolium salt (WST-1) (50 μM) and 1-methoxy-5-methylphenazinmethylsulphate (200 μM) for 3-h incubation. The absorbance was measured in microtitre plates at 450 nm with reference filter 690 nm by Spectra Rainbow (Tecan, Salzburg, Austria).

Determination of total cell ATP

ATP was measured both in cell lysate prepared in somatic cell ATP-releasing reagent (Sigma-Aldrich) and in cell lysate prepared for determining luciferase activity. Cells were washed by cold PBS and lysed in 400 μl of lysing reagent. Cell lysates were homogenized by pipetting and 50 μl was mixed with 25 μl of ATP reagent SL (Biothema, Haninge, Sweden). Luminescence was detected as described above for luciferase determination.

Statistical analysis

All the data are expressed as the means (±SEM) of at least three independent experiments. Calculations were performed with STATISTICA for Windows 7.0 (StatSoft, Inc., Tulsa, OK). Pearson correlation coefficient R and R-square (R^2) were used to determine the correlation between variables. Confidence intervals 0.95 were depicted in graphs.

Results

Different methods were employed to determine the number of viable cells. The measurement of ATP in cell lysate was compared with the measurement of the total amount of protein in cell lysate and with the measurement of WST-1 tetrazolium salt reduction (Fig. 1). All evaluated methods revealed a linear correlation with the number of seeded cells of both cell lines. The highest significance of correlation (Pearson correlation coefficient) was obtained comparing the seeded cell numbers with the luminescence-based ATP determination in P19 cells and the protein concentration in HT-29 cells. The

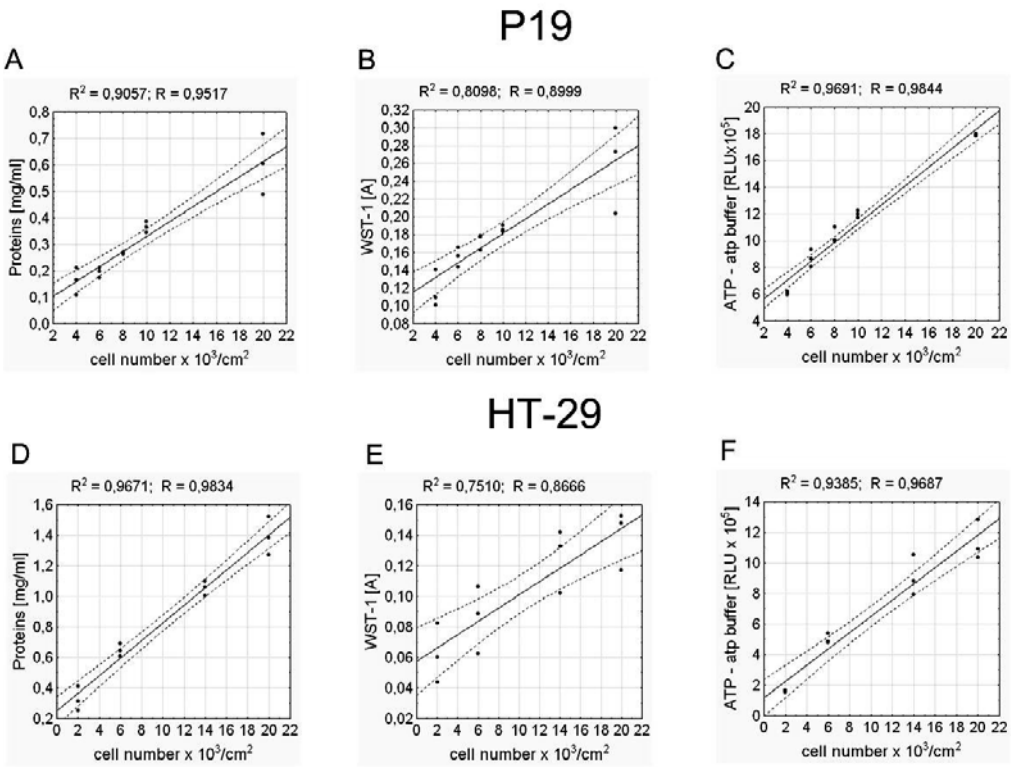

Fig. 1. Assessment of viable cells by colorimetric determination of total cell proteins in cell lysate (A, D), colorimetric determination of WST-1 reduction (B, E), and luminometric determination of intracellular ATP (C, F) in P19 and HT-29 cell lines. Pearson correlation coefficient R was used to determine the correlation between variables and the value of R-square (R^2) was calculated. Confidence intervals 0.95 are depicted in graphs.

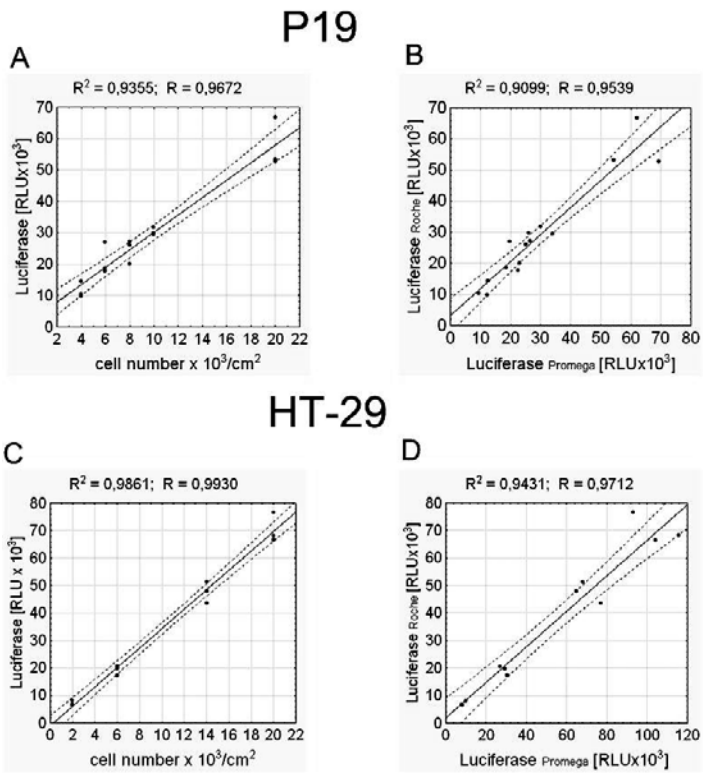

Fig. 2. Correlation between luciferase reporter gene activity and the number of seeded cells (A, C). Comparison of the luminiscence signal obtained from two different commercial kits (Luciferase Reporter Gene Assay and Luciferase Assay System) for determining luciferase activity (B, D). Pearson correlation coefficient R was used to determine the correlation between variables. The value of R-square (R^2) was calculated. Confidence intervals 0.95 are depicted in graphs.

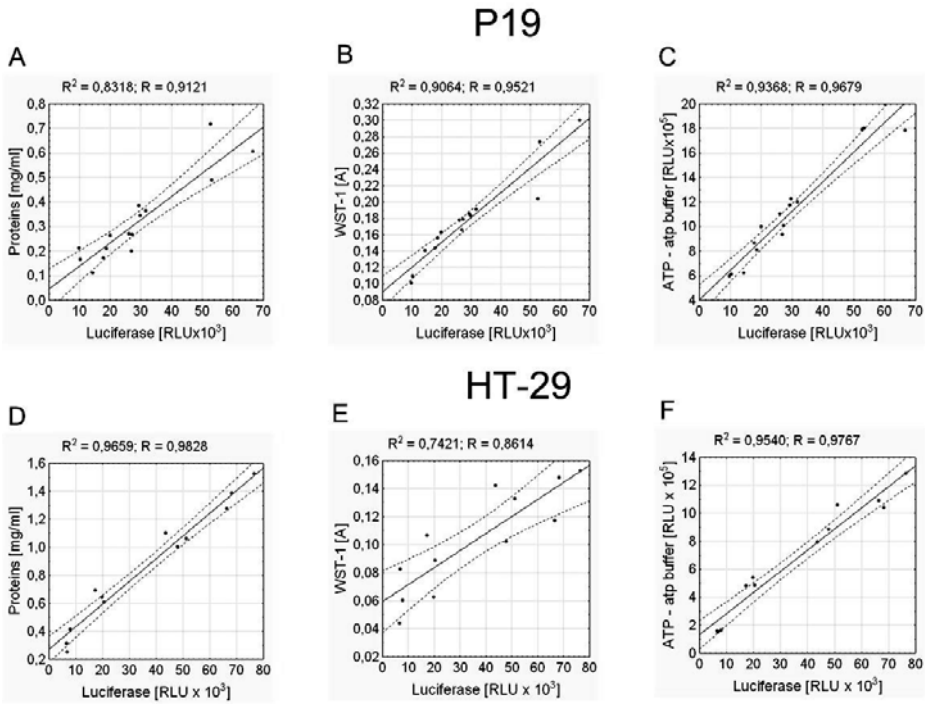

Fig. 3. Correlation between luciferase reporter gene activity and the amount of viable cells assessed by determination of total cell proteins in cell lysate (A, D), colorimetric determination of WST-1 reduction (B, E), and luminometric determination of intracellular ATP (C, F) in P19 and HT-29 cell lines. Pearson correlation coefficient R was used to determine the correlation between variables. The value of R-square (R^2) was calculated. Confidence intervals 0.95 are depicted in graphs.

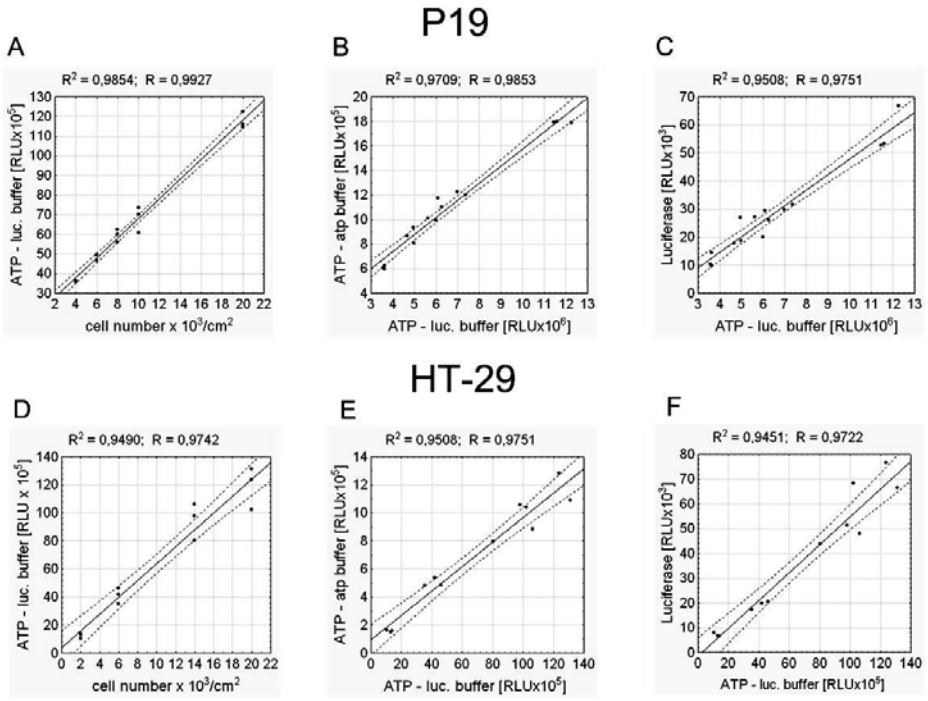

Fig. 4. Assessment of the amount of viable cells by determining the amount of intracellular ATP in whole-cell lysate prepared in lysing buffer originally designed for luminometric determination of luciferase activity [ATP luc buffer] (A, D). Correlation between luminometric determination of ATP in cell lysates prepared in two buffers, the ATP-releasing reagent [ATP-atp buffer] and the lysing buffer originally designed for luminometric determination of luciferase activity (B, E). Determination of luciferase activity in correlation with cell viability assessment by ATP determination in lysing buffer originally designed for luminometric determination of luciferase (C, F). Pearson correlation coefficient R was used to determine the correlation between variables. The value of R-square (R^2) was calculated. Confidence intervals 0.95 are depicted in graphs.

lowest correlation was obtained between the number of seeded cells and the WST-1 determination for both cell lines.

Luciferase activity in P19 cells activated by RA and HT-29 cells activated by TNF-α correlated significantly with the number of seeded cells (Fig. 2A and 2C). When comparing two different assays for luciferase determination (Roche and Promega, Fig. 2B and 2D), we observed high correlation between the luminescence signals. Both assays revealed a similar significance of correlation with the seeded numbers of both cell types (not shown). Further, luciferase activity correlated significantly with the assessment of viable cells by all of the three methods employed (Fig. 3). Interestingly, the ATP determination could also be performed in the cell lysates prepared in the lysing buffer that was designed for the determination of luciferase activity (Fig. 4A and 4D). Determination of ATP in this buffer for luciferase determination correlated significantly with the ATP determination performed in the lysing buffer "ATP-releasing agent" (Fig. 4B and 4E) and correlated significantly with the luciferase activity in RA-activated P19 cells and TNF-α-activated HT-29 cells (Fig. 4C and 4F). Similarly, luciferase activity could be determined in cell lysate prepared in the ATP-releasing agent, although with a reduced sensitivity: approximately 70 % compared with cells lysed in luciferase lysing buffers (not shown).

The ATP determination in the lysis buffer designed for determining the luciferase activity allowed determination of both ATP and luciferase activity in transiently transfected cells (Fig. 5A, 5B and 5C). Moreover, the results correlated explicitly with the enzymatic activity of β-galactosidase in the corresponding samples (Fig. 5D).

Further, the same transfection experiment was performed, but an identical cell number was seeded per well and various concentrations of RA were used. As expected, the luciferase activity mediated by pRAREβ2-TK-luc reporter increased in a RA dose-dependent manner; nevertheless, the ATP level, CMV-β-galactosidase activity and cell proteins were equal among the groups treated corresponding to the equal seeding cell number (not shown).

Discussion

When making correlations between experimental treatment and the expression of a reporter gene, other events associated with cell physiology are known to affect the reporter gene expression. Of particular concern is a cytotoxic effect which can resemble down-regulation of the reporter activity. Recently, two methodological approaches have been used to correct these unintentional effects on gene reporter assay activity and to avoid

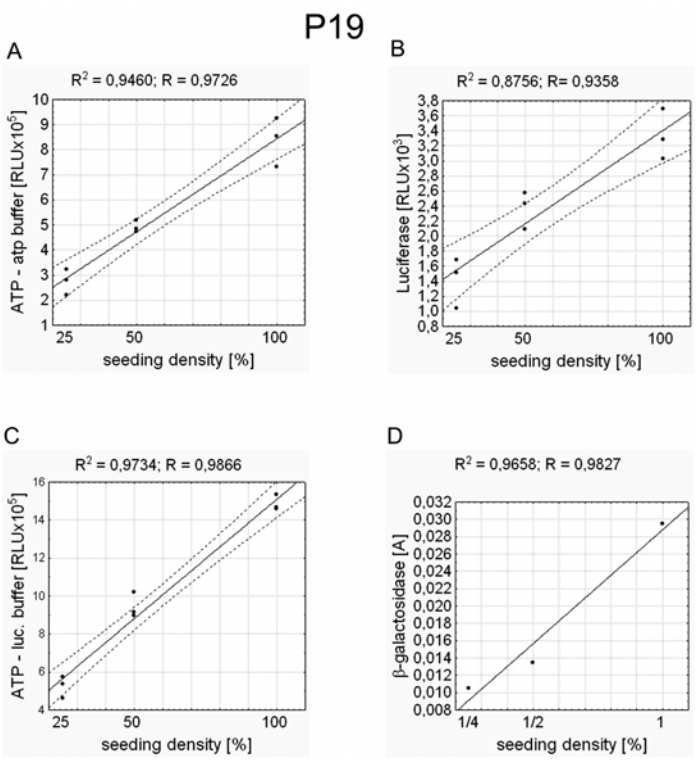

Fig. 5. Assessment of the amount of viable transiently transfected P19 cells by determining the amount of intracellular ATP in whole-cell lysate prepared in lysing buffer ATP-releasing reagent and lysing buffer originally designed for luminometric determination of luciferase activity (A, C). Luciferase activity in transiently transfected P19 cells by electroporation (B). The activity of β-galactosidase in transiently transfected P19 cells (D). Pearson correlation coefficient R was used to determine the correlation between variables. The value of R-square (R^2) was calculated. Confidence intervals 0.95 are depicted in graphs.

any misinterpretation of the data. The first approach involves independent monitoring of both reporter expression and cell viability; the second involves standardizing luciferase activity to another reporter vector carrying the gene for the reporter protein under the control of a constitutively active promoter. Normalizing an experimental reporter, such as firefly luciferase, with a constitutively active control reporter from the same sample, such as Renilla luciferase, allows a distinction to be made between specific and global effects on gene expression (Martin et al., 1996; Grentzmann et al., 1998). However, determination of a second reporter does not bring any new information about the cell viability, which could be significantly altered in transiently transfected cells. Further, selection of a suitable promoter directing the internal control reporter gene in transient transfection could be a complicated task. Further, in stably transfected cells the dual reporter system is not commonly used. Moreover, incorporation of additional genetic material to the genome may contribute to non-specific dysregulation of the gene expression and should be considered with caution when generating stably transfected cells (Rosenberg, 1997; Bryja et al., 2003; Wurtele et al., 2003). Nonetheless, determination of the cell viability cannot provide the researchers with the information regarding transfection efficiency.

Assessing viable cell numbers in a culture is a critical aspect in *in vitro* experiments. Commonly used cell viability assays include direct counting of live cells, intracellular ATP quantification, reduction of tetrazolium salts, and quantification of the esterase activity, nucleic acid, and total amount of proteins. Here, the ATP determination revealed the best correlation with the number of seeded cells. Interestingly, the determination of total cell lysate protein concentration could also be suggested as a suitable method for assessing the number of cells. However, the main advantage is that luminiscence-based ATP assessment can be readily combined with luciferase reporter assays.

In conclusion, the assessment of live cells by luminometric determination of ATP that can be multiplexed with a luciferase reporter gene assay allows independent monitoring of both reporter expression and cell viability. This approach avoids misinterpretation of data and allows more reliable reproducibility and reduction in the number of required sample replicates.

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