

Dual Cell-based Assay Protocols Applicable for Medium Throughput Screening

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Introduction

Anti-cancer drug screening on target proteins in higher throughput is primarily done with in-vitro assays, such as enzymatic or binding assays. Cytotoxicity assays are often added but it is difficult to draw conclusions on intracellular target-specific action. Reporter gene assays offer an alternative system of cell-based counter screening, which allows design of target-directed strategies to a certain degree. The desired target will rarely be a transcription factor that binds to a response element of a gene promoter and directly alters gene expression. Many targets will be involved in upstream signal transduction pathways ending in modification of a transcription factor. This opens up an opportunity to design indirect detection strategies for target activity. The response element is linked to a basal promoter in a reporter gene vector. In the nucleus of a transfected cell it will report the activity of the desired transcription factor and its control by the upstream signal transduction pathway(s). Transfection protocols have been developed in 96- and 384-well plate formats to increase throughput. However, when the desired cell line is difficult to transfect, the use of a stable line may be more appropriate for routine assays. In many applications drugs will be screened for repression of a signal transduction pathway, which may result in cell death, hence reductions in reporter gene activity need to be normalised for cytotoxicity or anti-proliferative effect. For transfection experiments normalisation is usually performed against a cotransfected, constitutively expressed second reporter. This takes alterations of transfection efficiencies into account or reports unrelated drug effects such as general transcriptional repression. When applying stable lines, an alternative standard has to be chosen, i.e. a cell-internal (endogenous) enzyme.

A convenient dual assay has been described [1] where endogenous acidic phosphatase was employed to normalise the subsequent β -galactosidase assay. The cell proliferation reagent WST-1 offers an excellent alternative to combine two or more assays on the same plate. Here we report on the development of protocols for normalised reporter gene assays suitable for medium throughput screening.

Materials and Methods

A single p53 response element was inserted downstream of the HSV tk basal promoter in a luciferase reporter gene plasmid. The reporter gene expression cassette was subcloned into pUB/bsd (Invitrogen). 5×10^5 A549-cells were transfected using $7 \mu\text{l}$ FuGENE (Roche Molecular Biochemicals) / $2 \mu\text{g}$ DNA / 6-well dish and were treated with $10 \mu\text{g/ml}$ blasticidin to select stable lines, which were named A-53 plus a serial number. T-22 cells [2] are a murine prostate derived line with stable expression of the β -galactosidase reporter gene linked to the p53 response element of the ribosomal gene cluster. For dual assays, cells were seeded into black-walled 96-well tissue culture plates with transparent bottom (ViewPlate-96, Black, Packard). For this and all subsequent steps, a growth medium without phenol red was used. Double concentrated drug solutions were prepared and added for 24 hours. Cell proliferation and reporter gene assays were performed as indicated in the protocols below. Reagents were prepared according to the supplier's instructions. Colour development of the WST-1 reagent was measured at 450 nm and the value of the no-cell control was subtracted. Relative light units of luciferase or β -gal activity were determined in a luminescence reader (TopCount, Packard) and the background levels measured before addition of the reagent were subtracted. Reporter gene activity was normalised by division with WST-1 absorption values.

Results and Discussion

A dual assay was developed for the β -galactosidase reporter gene cell line T-22. The protocol combined the determination of the reporter gene activity by the chemiluminescent β -gal assay kit with the cell proliferation reagent WST-1 (both Roche Molecular Biochemicals) for normalisation against mitochondrial reductase activity. T-22 cells were treated with Actinomycin D or H7, both previously shown to activate p53. Actinomycin D is a DNA intercalator that inhibits translation. H7 [1-(5-isoquinoline-sulfonyl)-2-methylpiperazine] is a widely used serine/threonine kinase inhibitor. After 24-hours treatment p53 induction was determined. The following protocol was used (all media was phenol red free).

Dual reductase/ β -galactosidase assay protocol

- Seed 8 000 T-22 cells / 50 μ l / well into 96-well plates.
- 24 hours later add 50 μ l double concentrated test compound diluted in growth medium.
- After 24-hours incubation add 100 μ l 20% WST-1 in growth medium (20 μ l WST-1 plus 80 μ l medium per well).
- After 30-minutes incubation read the colour development at 450 nm.
- Remove 200 μ l supernatant and wash once with PBS.
- Remove PBS and lyse cells with 50 μ l 1x Reporter Gene Assay Lysis Buffer while shaking for 30 minutes at RT.
- Add 100 μ l of the β -galactosidase Substrate Reagent and incubate for 15 minutes at RT.
- Add 50 μ l Initiation Reagent.
- Incubate for 2 minutes in the dark inside the luminescent counter and read light units.

We have tested incubation times with WST-1 over a period of 30 minutes and observed that the longer the incubation time the lower the variance. In step f) 100 μ l Lysis Buffer may be applied to determine protein concentration in 50 μ l cell lysate after a replication step. A copper-based protein determination kit is recommended due to ingredients in the Lysis Buffer.

Induction of p53 was demonstrated for both compounds (Figure 1), although the observed profiles differed from each other. Error bars are indicated for the data points determined from triplicate plating.

For assays with the reporter line A-53#33 the WST-1 reagent was combined with the Roche Luciferase Reporter Gene Assay, Constant Light Signal. The assay was established using treatment with Actinomycin D. The following protocol was derived (all media were phenol red free).

Dual reductase/luciferase assay protocol

- Seed 12 000 A-53#33 cells / 50 μ l / well.
- 24 hours later add 50 μ l double concentrated test compound.
- After 24-hours incubation add 100 μ l 20% WST-1 reagent in growth medium.
- After 30 -minutes incubation read absorbance at 450 nm.
- Remove 200 μ l supernatant and wash once with medium.
- Add 100 μ l growth medium and determine background in the luminescence counter.
- Add 100 μ l Luciferase Substrate Reagent.
- Incubate for 2 minutes in the dark inside the luminescent counter and read light units.

For validation of this assay, three 96-well plates were treated with 30 ng/ml Actinomycin D per well. Results were

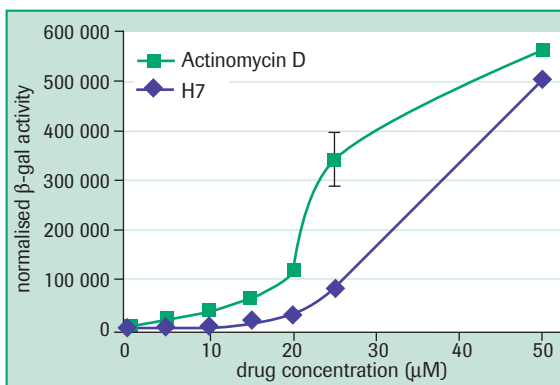


Figure 1: p53-Induction in T-22 cells after 24-hours treatment with Actinomycin D or H7

Table 1: Validation of dual reductase/luciferase assay

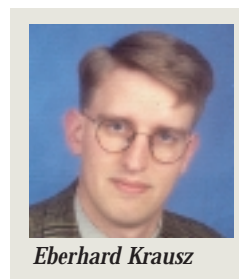
| | RLU/WST-1 minus background | |
|------|----------------------------|--------------------|
| | Basal expression | Induced expression |
| Mean | 272.35 | 5739.06 |
| SD | 27.60 | 566.01 |
| %CV | 10.14 | 9.85 |

(SD = standard deviation, CV = coefficient of variation)

compared to three untreated plates (Table 1). A signal over background ratio of 21 was determined.

Summary

The dual assay protocols have been developed for screening of p53 induction, which are less complex and more rapid in comparison to previous individual assays. The relatively low number of pipetting steps make the dual reductase/luciferase or β -galactosidase assays very convenient for medium throughput applications and form the basis for further development towards higher throughput.



A critical factor for further improvement could be the relatively rapid colour development of the WST-1 reagent while the light signals are stable with half-lives of 3.5 - 4.5 hours (luc reagent) or 1.5 hours (β -gal reagent). The lower number of steps in the dual reductase/luciferase assay makes this protocol more attractive for further development.

References

- Groth D. et al. (1998), *Anal. Biochem.* 258: 141-143.
- Böttger A. et al. (1997), *Curr. Biol.* 7: 860-869.

| Product | Pack Size | Cat. No. |
|---|-------------------|-----------|
| Cell Proliferation Reagent WST-1 | 2500 tests | 1 644 807 |
| Luciferase Reporter Gene Assay, Constant Light Signal | 1000 tests | 1 897 667 |
| β -Gal Reporter Gene Assay, chemiluminescent | 1 kit (500 tests) | 1 758 241 |
| Reporter Gene Assay Lysis Buffer | 50 ml | 1 897 675 |

