

A protocol for enhancement of the AAV-mediated expression of transgenes

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1. Introduction

This protocol describes a method for enhancing AAV vector-mediated gene expression by gamma-ray irradiation (as outlined schemetically in Fig. 1).

2. Background and Principle

AAV is widely used as a vector for the transfer of genes into a variety of cells including human cells. In some cases, it is desirable to enhance the AAV-mediated expression of transgenes to achieve specific goals.

Genotoxic stress, such as irradiation with gamma-rays or the application of topoisomerase inhibitors, improves transgene expression¹ as a result of the enhancement of second-strand synthesis of the rAAV genome.

We describe here the procedure for the enhancement of the expression of LacZ, an example.

3. Reagents

293 cells (human embryonic kidney cells)

NKO-1 cells (squamous cell carcinoma cells)

Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12)

Fetal bovine serum

AAV vector encoding LacZ

5-Bromo-4-chloro-3-indolyl β -D-galactoside (X-gal)

β -Gal Assay kit (Roche, Germany)

Phosphate-buffered saline (PBS)

Solution of 0.05% glutaraldehyde in PBS

0.5 M $K_3[Fe(CN)_6]$

0.5 M $K_4[Fe(CN)_6]$

1 M $MgCl_2$

0.25 M Tris-Cl (pH 8.0)

Solution of 4 mg/ml ONPG in water

Reaction buffer containing 0.6 M Na₂HPO₄, 0.4 M NaH₂PO₄, 0.1 M KCl, 0.01 M MgSO₄, and 40 mM β-mercaptoethanol (pH 7.0)

1 M Sodium carbonate

4. Methods

4.1. Preparation of cells

Culture 293 and NKO-1 cells in DMEM/F-12 with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂ in air. One day before transduction, plate 1x10⁶ cells in individual 10-cm dishes.

4.2. Irradiation of cells

Irradiate cells with 1-4 Gy, as indicated by the manufacturer of the irradiation device, immediately before transduction to achieve the strongest enhancement of transgene expression.

4.3. AAV-mediated transfer of genes to cultured cells

Prepare an AAV vector that harbors the *LacZ* gene from *Escherichia coli* as described previously. Add 2 x 10¹⁰ copies of AAV-LacZ to the culture medium in a single 10-cm dish. After transduction, return cells to the incubator at 37°C.

4.4. Detection of β-galactosidase activity

Thirty-six hours after transduction with AAV-LacZ, evaluate the expression of the transgene by at least one of the following procedures.

4.4.1. Staining with X-gal

After removal of the culture supernatant by aspiration, fix cells in 0.05% glutaraldehyde in PBS for 5 minutes at room temperature. Wash cells twice with PBS. Stain cells with X-gal (0.64 mg/ml) in PBS that contains 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], and 1 mM MgCl₂ for more than 6 hr. Determine the efficiency of transduction from the relative number of blue-stained cells.

4.4.2. Quantitative analysis of the expression of the β-galactosidase

The amount of β-galactosidase expressed can be determined by monitoring the hydrolysis of ONPG. A convenient kit is available from Roche as a "β-gal ELISA kit".

1. Wash the monolayer of transfected cells once with PBS.
2. Harvest the cells.

3. Centrifuge the suspension of cells at 250 x g for 5 minutes. Remove the supernatant by aspiration.
4. Resuspend the pellet of cells in 0.25 M Tris-Cl (pH 8.0) at 4°C.
5. Freeze and thaw the suspension twice.
6. Centrifuge at 15,000 x g for 5 min. Transfer 10 µL of the supernatant to a new tube.
7. Add 20 ml of distilled water, 70 µL of a solution of 4 mg/ml ONPG, and 200 µL of reaction buffer. Incubate at 37°C for 30 min. To stop the reaction, add 500 µL of 1 M sodium carbonate.
8. Determine the absorbance at 420 nm relative to that of a blank sample (prepared without cell lysate).
9. Determine the concentration of protein in the cell lysate and calculate the specific activity of β-galactosidase.

4.5. Evaluation of enhancement

Plot the results obtained in 4.4. Compare relative numbers of positive cells or β-gal activity in terms of the dose of vector or of gamma-irradiation.

Typical results are shown in Figure 2.

5. Notes

1. The effects of the timing of irradiation on the efficiency of transduction are indicated in Figure 2. Transduction of NKO-1 cells at 6×10^4 particles/cell was performed immediately before (open squares), immediately after (closed squares), and 6 hr. after (open circles) irradiation. When transduction was performed more than 6 hr. before or more than 12 hr after irradiation, the irradiation had no effect on the expression of LacZ.
2. The enhancement of transgene expression is related to increases in the rate of second-strand synthesis, which can be confirmed by Southern analysis of the transduced cells.
3. The protocol described above allows enhancement of the expression of LacZ, as an example. This protocol can be used to enhance the expression of other genes. The efficacy of this system was validated in a well-studied suicide-

gene system (HSV tk combined with ganciclovir) in which the enhancement of tumoricidal activity was demonstrated.²

6. References

1. Russell DW, Alexander IE, Miller AD. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. *Proc Natl Acad Sci USA*. 1995; 92: 5719-5723.
2. Kanazawa T, Urabe M, Mizukami H, Kume A, Nishino H, Monahan J, Kitamura K, Ichimura K, Ozawa K. γ -rays enhance the transduction efficiency with rAAV and the cytotoxic effect of AAV-HSV tk /ganciclovir on cancer cells. *Cancer Gene Therapy*. 2001; 8: 99-106.

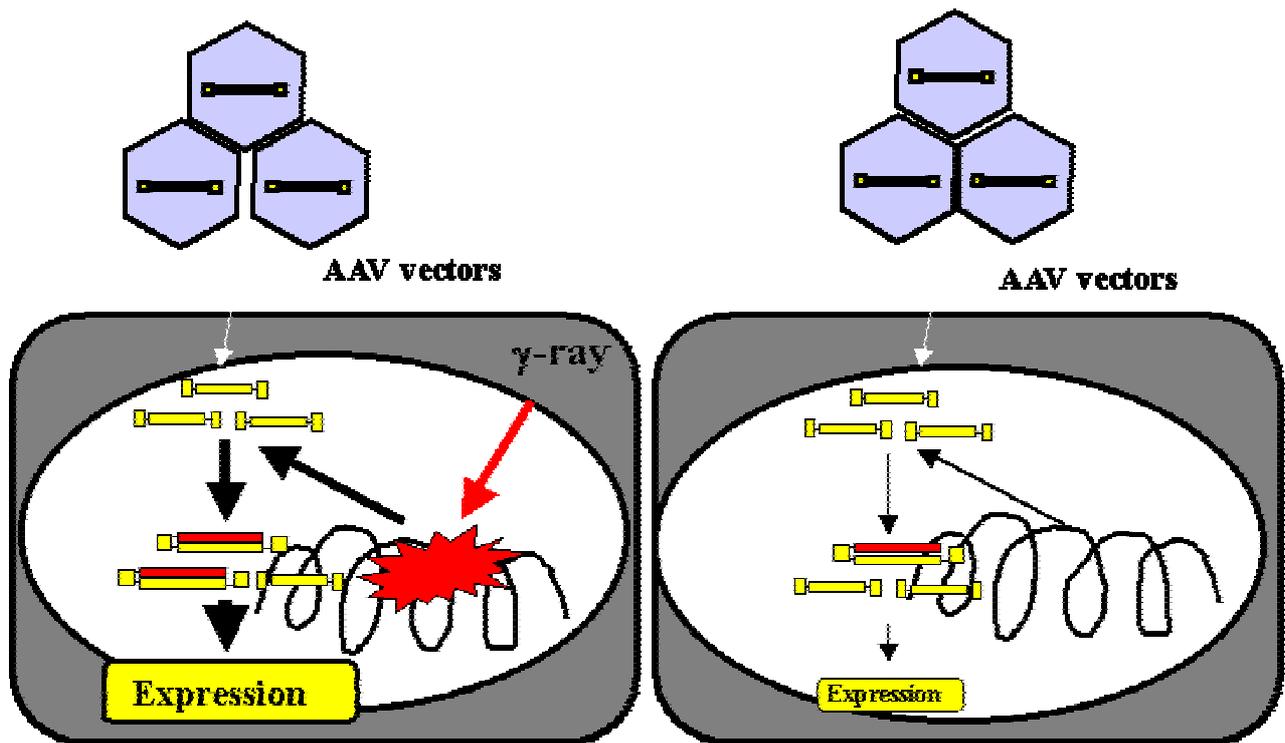


Fig.1 Enhancement of AAV-mediated transgene expression by γ -ray

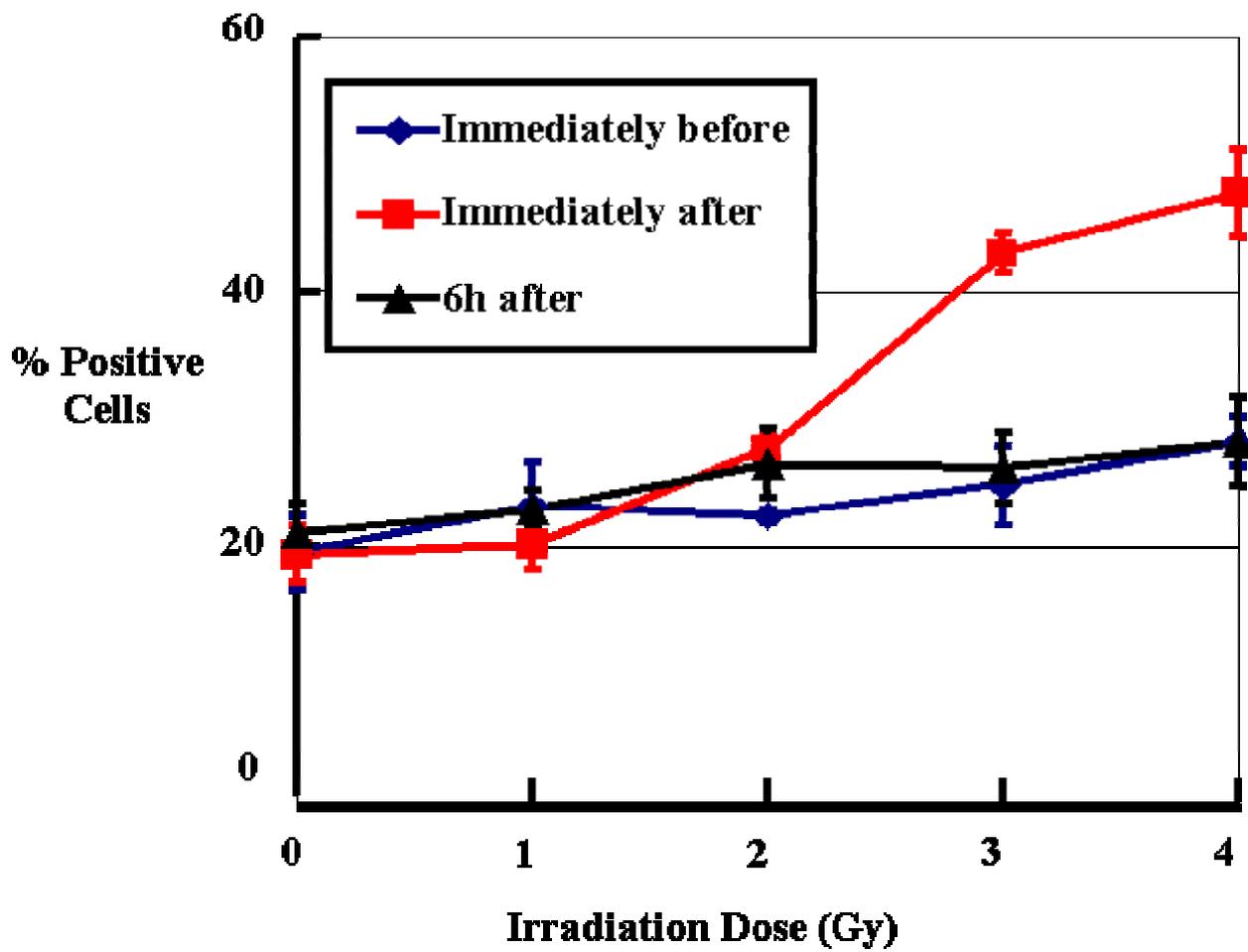


Fig.2 Effect of irradiation timing relative to transduction upon transgene expression