SHORT TECHNICAL REPORTS

Manuscripts published in the Short Technical Reports section are shorter and less comprehensive in scope than full Research Reports.

Herpes Simplex Virus Thymidine Kinase-Green Fluorescent Protein Fusion Gene: New Tool for Gene Transfer Studies and Gene Therapy

BioTechniques 24:614-618 (April 1998)

ABSTRACT

Green fluorescent protein (GFP) and herpes simplex virus type-1 thymidine kinase (TK) are commonly used markers in gene transfer studies. The latter gene has also proven to be an effective tool in cancer "suicide" gene therapy. To facilitate rapid and reliable selection of cells expressing TK, we constructed a plasmid expressing a TK-green fluorescent protein fusion gene (TK-GFP). In this fusion gene, the expression of each component is coupled to one another, permitting accurate determination of the percentage of cells expressing TK by detecting the green fluorescence produced by GFP. Transfection of the fusion plasmid to mammalian cells revealed that the construct is fully functional, making the cells both fluorescent and sensitive to ganciclovir.

INTRODUCTION

The 27-kDa green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* emits a characteristic fluorescence when exposed to long wavelength UV light and has been used as a reporter of gene expression and as a fusion tag to study protein localization in a wide variety of living cells (5,11, 15,17). Herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) selectively converts the nontoxic pro-drug ganciclovir (GCV) into a toxic phosphorylated derivative. Expression of viral TK in eukaryotic cells causes cell death upon exposure to GCV (6). TK has been used both as a negative in vitro selection marker and as a "suicide gene" in cancer therapy.

The inability to accurately measure the transduction efficiency of the therapeutic gene is a major factor in the difficulty in predicting the efficiency of a viral vector (16). In the case of retroviral vectors, gene transfer efficiency is a crucial factor to determine success of an in vivo trial. This information has often been overlooked, and the estimations of transduction efficiency have solely been based on the results of therapy. Most commonly, attempts to determine the actual rate of gene transfer have used two vectors, one carrying the therapeutic gene and the other a marker gene. Another approach is to use vectors with two expression cassettes, each driven by different promoters. The third possibility is to use a common promoter for two expression units that are separated by an internal ribosomal entry site (IRES). All these approaches, however, are hampered by the fact that the expression level of the marker gene can be dramatically different than the therapeutic gene.

In this study, we created a combined marker gene-therapeutic gene system to circumvent these problems in determining TK gene transfer efficiency before GCV therapy. We constructed a fusion gene system containing both TK and GFP in the same transcription unit and driven by the same promoter. This construct produces functional fusion protein that has the activities of both components, making the target cells sensitive to GCV and emitting green fluorescence under UV light.

MATERIALS AND METHODS

Plasmid Constructs

The TK and GFP expression plasmids were constructed by ligating the 1.2-kb DNA fragment encoding the TK gene (12) or cycle-3 GFP mutant (4) into mammalian expression vector pEF-BOS (14). The TK-GFP fusion gene was constructed by inserting both TK and GFP fragments into pEF-BOS, yielding plasmid pETGB. To create an in-frame connection between the two coding sequences, a double-stranded oligonucleotide linker was inserted between TK and GFP genes in pETGB to make pETLGB. Figure 1 shows the structure of pETLGB. The plasmid pNEO was constructed in pBluescript® (Stratagene, La Jolla, CA, USA) by inserting an expression cassette simian

virus 40 (SV40) promoter, neomycin phosphotransferase gene and polyadenylation signal from the plasmid pLXSN (13).

Cell Culture

TK-deficient Rat2 fibroblasts (Catalog No. CRL-1764; ATCC, Rockville, MD, USA) and BT4C rat glioma cells (10) were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (both from Life Technologies, Paisley, Scotland, UK), 2 mM glutamine, 2 mM sodium pyruvate and 50 µg/mL gentamicin at 37°C in the presence of 5% CO_2. Transfections were performed using calcium phosphate-DNA precipitation (2). Cells (10^5) were seeded into 6-well plates 24 h before the transfection. The cells were incubated with 5 µg of plasmid DNA, including 0.75 µg pNEO plasmid, for 8 h. Selection medium containing Geneticin® (G418) or hypoxanthine-aminopterin-thymidine (HAT) (both from Sigma Chemical, St. Louis, MO, USA) was added 48 h post-transfection to BT4C+TK, BT4C+TK-GFP, BT4C+GFP and Rat2+GFP cells or to Rat2+TK and Rat2+TK-GFP cells, respectively. The G418- or HAT-resistant colonies from each transfection were pooled and expanded into stable cell lines.

RNA and Protein Analysis

For RNA analysis, cultured cells were trypsinized, washed with phosphate-buffered saline (PBS), and total RNA was isolated using acidic guanidium thiocyanate/phenol/chloroform extraction (3). The RNA samples (8 µg) were denatured, and Northern blot was carried out using TK-specific, digoxigenin-labeled probe. Chemiluminescent detection was performed as described (7) using CDP-Star™ (Boehringer Mannheim GmbH, Mannheim, Germany) as a substrate.

For protein analysis, collected cells (2×10^6) were resuspended in 40 µL of lysis buffer (25 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) and lysed by sonication at 4°C. The lysates were clarified by centrifugation at 14 000× *g* for 5 min. Ten microliters of lysates were electrophoresed in 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto Immobilon®-P Polyvinylidene Difluoride Membranes (Millipore, Bedford, MA, USA). Membranes were blocked and incubated with rabbit polyclonal anti-TK antibody (W.C. Summers, Yale University, New Haven) or mouse monoclonal anti-GFP antibody (CLONTECH Laboratories, Palo Alto, CA, USA). After washing, the membranes were incubated with goat anti-rabbit IgG-AFOS or with rabbit anti-mouse IgG-AFOS (both from Zymed Laboratories, San Francisco, CA, USA) and developed using nitro blue tetrazolium/Br(Cl)Ind-P staining.

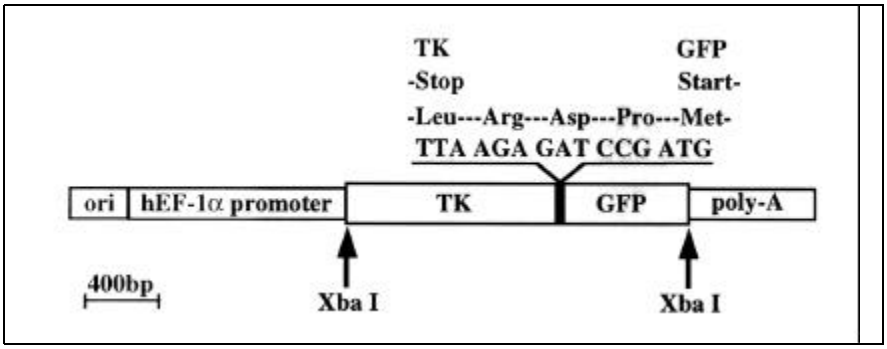

Figure 1. The TK-GFP fusion gene plasmid (pETLGB). The expression cassette contains the SV40 replication origin (ori), the human polypeptide chain elongation factor 1α promoter (hEF-1α) and the polyadenylation signal [poly(A)] from human granulocyte colony stimulation factor cDNA. The fusion of TK and GFP was carried out by replacing the TK stop codon with a linker sequence shown above the gene construct.

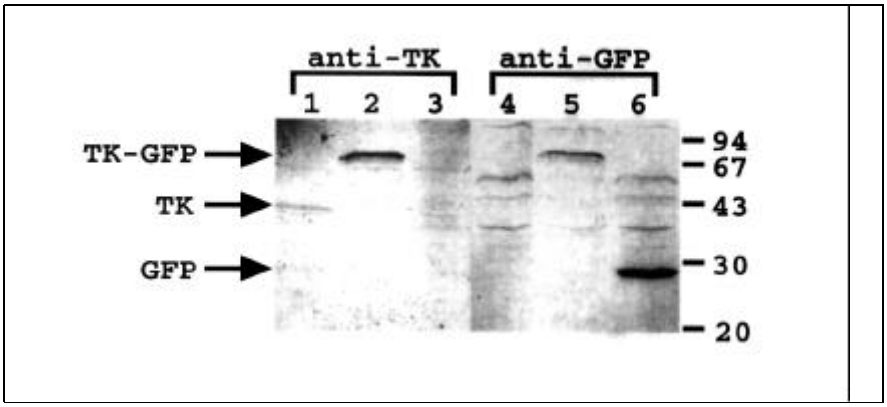

Figure 2. Immunoblot analysis of transfected Rat2 cells using TK- (lanes 1–3) or GFP- (lanes 4–6) specific antibody. Protein samples were electrophoresed in SDS polyacrylamide gel electrophoresis gels and transferred to Immobilon P for immunoblot analysis. Lanes 1 and 4, TK; lanes 2 and 5, TK-GFP; and lanes 3 and 6, GFP. Molecular mass markers (in kDa) are shown to the right.

Thymidine Kinase Enzyme Activity

Cells (2×10^6) were homogenized as described above. Five microliters of 1:25 dilution of the lysates were added to 45 µL of reaction mixtures containing 100 mM Tris-HCl, pH 7.5, 10 mM ATP, 7 mM $MgCl_2$ and 1.7 µM [^{3}H]thymidine (21 Ci/mmol). The mixtures were incubated for 30 min at 37°C, and reactions were stopped, incubating samples at 98°C for 2 min. Aliquots (25 µL) were pipetted onto DE-81 paper (Whatman International Ltd, Maidstone, England, UK), washed twice in 10 mM Tris-HCl, pH 7.5 and three times in 95% ethanol then air-dried. [^{3}H]thymidine monophosphate produced was measured by liquid scintillation counting (1).

Ganciclovir Sensitivity and Determination of GFP Expression

Polyclonal populations of TK-, TK-GFP- and GFP-transfected Rat2 cells (2×10^4) were seeded into 6-well plates and incubated in the presence of differ-

ent doses of GCV (0, 0.003, 0.01, 0.03, 0.1, 1, 10 and 100 μg/mL) for four days. The cells were trypsinized and counted.

GFP expression was monitored in living Rat2 and BT4C cells using a Diaphot 300 Inverted Microscope (Nikon, Melville, NY, USA) equipped with a V-2A filter set. Cells were grown in 6-well plates, and growth media was changed to PBS before photography. 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Boehringer Mannheim) staining was done according to the manufacturer's instructions.

RESULTS

Expression of the TK-GFP Fusion Gene

Detection of the appropriate sized transcripts and peptides of the TK-GFP fusion gene was carried out by analyzing the size of the mRNA with Northern blotting and molecular weight comparison by Western blotting. The RNA with digoxigenin-labeled TK DNA probe showed a 1.9-kb band in TK cells and a 2.6-kb band in TK-GFP cells. These signals corresponded with the predicted mRNA lengths for the respective expression plasmids (results not shown).

Immunoblot analysis using rabbit TK-specific polyclonal antibody revealed a 43-kDa band in Rat2+TK cells and a 70-kDa band in Rat2+TK-GFP cells. These signals also corresponded with the predicted sizes of TK and the TK-GFP fusion peptide. Immunoblot with mouse GFP-specific monoclonal antibody showed a 27-kDa band in a Rat2+GFP sample and a 70-kDa band in a Rat2+TK-GFP sample (Figure 2).

Activity of the TK-GFP Fusion Protein In Vitro

The function of the fusion protein was examined by green fluorescence, TK activity and GCV sensitivity in stably transfected cell populations. BT4C cells expressing GFP, TK-GFP or TK were examined by fluorescence microscopy. As shown in Figure 3, cells expressing the fusion construct emitted green light, but the distribution of the fluorescence produced by the fusion protein was different from that of the native GFP (4), being more localized in the nucleus. The nuclear localization was also confirmed with DAPI staining (results not shown).

TK activity of cells expressing each construct was tested by an enzymatic assay for phosphorylated [^{3}H]thymidine absorbed to DE-81 paper. The average enzyme activity of the TK-GFP fusion protein was 80% of the native TK protein. Rat2 or Rat2+GFP cells showed no TK activity (results not shown).

Cells expressing TK, TK-GFP or GFP were treated with increasing doses of GCV for four days, and the number of cells was determined. Both TK and TK-GFP expressing cells showed similar sensitivity to GCV, although there was a slight difference with the lowest concentrations of GCV (Figure 4).

DISCUSSION

We have constructed a TK-GFP fusion gene encoding a bifunctional protein that contains both TK and GFP activities. Cells expressing the fusion protein can be detected under UV light by green fluorescence, and they are highly sensitive to GCV.

When the activities of the two domains of the TK-GFP fusion protein were compared with constructs containing only TK or GFP, the TK-GFP fusion protein was virtually as effective as TK alone. A slight difference in TK activity and GCV sensitivity suggest

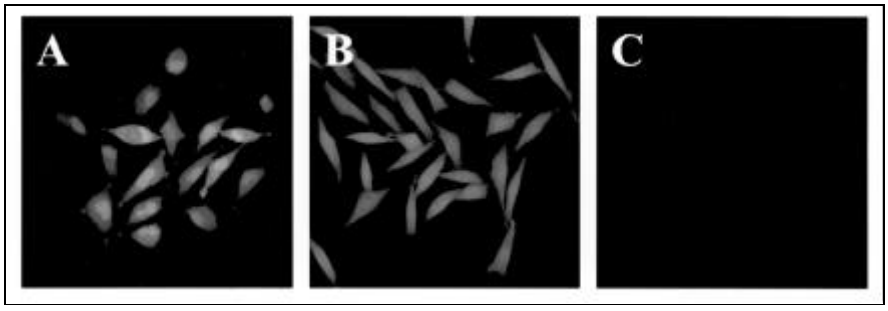

Figure 3. Fluorescence microscopy images of BT4C cells transfected with the plasmids encoding (A) TK-GFP, (B) GFP and (C) TK. (A) Cells transfected with the fusion construct TK-GFP display an expression mostly concentrated in the nucleus. (B) Expression of normal GFP can be seen throughout the cells. (C) TK-transfected cells show no fluorescence. (Magnification, ×200)

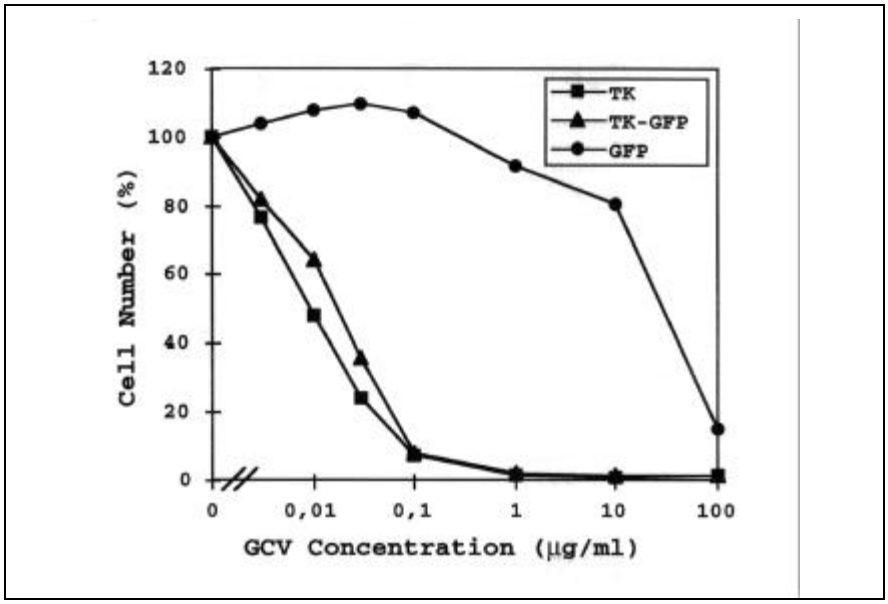

Figure 4. Toxicity of GCV to Rat2 cells transfected with TK, TK-GFP or GFP. Cells were treated with the indicated concentrations of GCV for 4 days. The proliferation of cells not treated with GCV was defined as 100%. The assay was carried out in duplicates.

that the GFP domain of the fusion protein was mildly inhibitory to the function of the TK domain, possibly by altering the optimal conformation of the polypeptide or by creating steric hindrance, thus making the active site of the TK domain less accessible to the substrate.

The cellular pattern of the green fluorescence from the fusion protein was different from that of the native GFP, having a greater localization of the fusion protein over the nuclear region. In the case of the fusion gene, the major intracellular expression site may be governed by the first domain, i.e., TK. There is currently very little data available about the site of expression of this protein, and only two reports have been published. The earlier report stated that TK was localized to the nucleus (9). This study, however, did not include any comment about the cytoplasmic expression. The more recent report indicated that in HSV-1-infected cells most of the TK expression occurred in the cytoplasm, especially in the perinuclear region (8). Due to limited and conflicting data about the expression site of TK, it is difficult to assess whether the different expression pattern of the fusion protein is related to the fusion structure itself, the natural expression site of TK, or whether this is a cell-line-specific feature. Experiments are currently in progress to answer these questions, using an HSV-TK-specific antibody in different TK-expressing cell lines.

Use of a TK-GFP fusion gene for in vitro clone selection in TK-negative cell lines would provide an easy means to select the best expressing clones based on the intensity of green fluorescence. The TK-GFP fusion protein also offers the possibility for easy control of long-term expression of transferred genes in established cell lines. For gene transfer studies in vivo, it provides a means to directly determine the transduction efficiency, to remove the GFP-expressing cells with GCV and, most importantly, to determine the actual minimal gene transfer efficiency required for total destruction of the tumor target.

REFERENCES

1. **Chen, C., Y. Chang, P. Ryan, M. Linscott, G. McGarrity and Y.L. Chiang.** 1995. Effect of herpes simplex virus thymidine kinase expression levels on ganciclovir-mediated cytotoxicity and "bystander effect". Hum. Gene Ther. *6*:1467-1476.
2. **Chen, C. and H. Okayama.** 1987. High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. *7*:2745-2752.
3. **Chomczynski, P. and N. Sacchi.** 1987. Single-step method of RNA isolation by acidic guanidium thiocyanate-phenol-cloroform extraction. Anal. Biochem. *162*:156-159.
4. **Crameri, A., E.A. Whitehorn, E. Tate and W.P.C. Stemmer.** 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nature Biotechnol. *14*:315-319.
5. **Cubitt, A.B., R. Heim, S.R. Adams, A.E. Boyd, L.A. Gross and R.Y. Tsien.** 1995. Understanding, improving and using green fluorescent proteins. Trends Biochem. Sci. *20*:448-455.
6. **Elion, G.B., P.A. Furman, J.A. Fyfe, P. DeMiranda, L. Beauchamp and H.J. Schaeffer.** 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. Proc. Natl. Acad. Sci. USA *74*:5716-5720.
7. **Engler-Blum, G., M. Meier, J. Frank and G.A. Müller.** 1993. Reduction of background problems in nonradioactive Northern and Southern blot analyses enables higher sensitivity than ^{32}P-based hybridizations. Anal. Biochem. *210*:235-244.
8. **Haarr, L. and T. Flatmark.** 1987. Evidence that deletion of coding sequences in the 5′ end of the thymidine kinase gene of herpes simplex virus type 1 affects the stability of the gene products. J. Gen. Virol. *68*:2817-2829.
9. **Herzberg, M., V. Bibor-Hardy and R. Simard.** 1981. Herpes specific and alpha DNA polymerase in nuclear envelope of BHK infected cells. Biochem. Biophys. Res. Commun. *100*:644-650.
10. **Laerum, O.D., M.F. Rajewsky, M. Schachner, D. Stavrou, K.G. Haglid and A. Haugen.** 1977. Phenotypic properties of neoplastic cell lines developed from fetal rat brain cells in culture after exposure to ethylnitrosourea in vivo. Z. Krebsforsch *89*:273-295.
11. **Levy, J.P., R.R. Muldoon, S. Zolotukhin and C.J. Link, Jr.** 1996. Retroviral transfer and expression of a humanized, red-shifted green fluorescent protein gene into human tumor cells. Nature Biotechnol. *14*:610-614.
12. **McKnight, S.L.** 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res. *8*:5949-5964.
13. **Miller, A.D. and G.J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques *7*:980-990.
14. **Mizushima, S. and N. Nagata.** 1990. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res. *18*:5322.
15. **Muldoon, R.R., J.P. Levy, S.R. Kain, P.A. Kitts and C.J. Link, Jr.** 1997. Tracking and quantitation of retroviral-mediated transfer using a completely humanized, red-shifted green fluorescent protein gene. BioTechniques *22*:162-167.
16. **Smiley, W.R., B. Laubert, B.D. Howard, C. Ibanez, T.C. Fong, W.S. Summers and F.J.**

Burrows. 1997. Establishment of parameters for optimal transduction efficiency and antitumor effects with purified high-titer HSV-TK retroviral vector in established solid tumors. Hum. Gene Ther. *8*:965-977.

17. **Zolotukhin, S., M. Potter, W.W. Hauswirth, J. Guy and N. Muzyczka.** 1996. A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. J. Virol. *70*:4646-4654.

We are grateful to Dr. John Morris for critical reading of the manuscript. We thank Dr. W.C. Summers for kindly providing the TK-specific antibodies. Address correspondence to Sami Loimas, A.I.Virtanen Institute, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland. Internet: loimas@messi.uku.fi

Received 28 August 1997; accepted 31 December 1997.

Sami Loimas[1], Jarmo Wahlfors[1,2] and Juhani Jänne[1]
[1]University of Kuopio
Kuopio, Finland
[2]National Human Genome Research Institute, NIH
Bethesda, MD, USA

Quantitative RT-PCR Amplification of RNA in Single Mouse Oocytes and Preimplantation Embryos

BioTechniques 24:618-623 (April 1998)

ABSTRACT

*We describe a simple whole-cell method for quantitative reverse transcription (RT) PCR amplification of RNA that consistently allows the analysis of trace amounts of RNA, such as those carried by a fraction of a single mouse oocyte or preimplantation embryo, without organic extraction. The method is based on a preliminary genomic DNA digestion by DNase I in the presence of Mn^{++} and a subsequent RT step with rT*h *Reverse Transcriptase at 70°C with the same buffer components, which also has the effect to irreversibly denature DNase I activity. Because of the completeness of genomic DNA digestion and RNA recovery, this procedure makes it possible to quantitatively amplify any target RNA, including those coded by intronless genes or genes whose intron-exon boundaries are unknown. By taking mRNAs of b-actin, heat-shock protein HSP70.1 and ribosomal protein S16 as experimental models, we demonstrate the effectiveness of genomic DNA digestion by DNase I-Mn^{++} and of DNase I heat-denaturation and the quantitative properties of our method. We also show that this procedure is useful for transcriptional analyses during development that are hindered by paucity of biological material.*

INTRODUCTION

Because of its sensitivity, reverse transcription-polymerase chain reaction (RT-PCR) RNA amplification has rapidly become the method of choice for detecting and measuring rare messages. This procedure can also be scaled down to the level of a few or single cells (17) and performed on crude whole-cell lysates (12). In the latter case, RNA recovery is complete, but the presence of genomic DNA may give false-positive signals. This problem can be overcome by different strategies. First, oligodeoxyribonucleotide primers can be designed appropriately to span an intron and thus produce different-sized DNA and RNA amplification bands. Unfortunately, this strategy requires the genes whose transcript is examined to have been cloned and sequenced. It thus cannot be applied when intron-exon boundaries are still unknown or when the RNA to be amplified is coded by an intronless gene. This is the case, among others, of many heat-shock genes. Second, amplification of RNA, but not DNA, can be achieved by the use of a poly(dT) antisense primer that anchors to the message's poly(A) tail (6). In most cases, however, such strategy limits message amplification to the few hundreds of nucleotides that are close to the poly(A) tail, leaving the message 5′ region virtually inaccessible to investigation. Third, RNA preparation can be preliminarily treated with DNase. Problems in this case will include the control of DNase digestion completeness and the removal/inactivation of the enzyme before the RT step. This is usually performed by extracting the DNase I-treated lysate with organic solvents or by using bead-immobilized DNase I (16,17). In both cases, however, RNA recovery may not be complete because of the necessary transfer of the supernatant/column eluate to another tube.

We have solved this problem by treating the crude, whole-cell lysate with pancreatic DNase I in the presence of Mn^{++} (2) and then irreversibly inactivating this enzyme with the 70°C incubation needed for RT by rT*th* Reverse Transcriptase. In this paper, such strategy has been optimized for quantitative RNA analysis on mouse oocytes and preimplantation embryos on a single-cell basis. This was particularly worth pursuing because, in spite of the relevance that these cells have for both basic and clinical research in humans and domestic mammals, to our knowledge, no RT-PCR method allowing message analysis to be performed on individual mammalian oocytes or early embryos has been reported so far. In fact, previous RT-PCR studies on these cells (9,13–15) used pools of 40–200 oocytes or embryos and were mostly based on RNA extraction-precipitation procedure. We show here that our procedure is consistent, quantitative and sensitive enough to determine the