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Isolation and Quantification of Episomal Expression Vectors in Human T Cells

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Isolation of low-molecular-weight DNA from eukaryotic cells entails the separation of a very small quantity of plasmid DNA from a much larger quantity of genomic DNA. Traditionally, the Hirt extraction method (3) has been used for this purpose. This method, however, is labor-intensive, involving extensive precipitations and phenol/chloroform extractions. In this report, three alternative methods are compared for the isolation of episomal cDNA expression vectors containing the human interleukin-2 (*IL-2*) or interferon-γ (*IFN-g*) gene from T lymphocytes that were stably transfected with the corresponding constructs: *(i)* a boiling method (4), *(ii)* alkaline lysis based on the method of Birnboim and Doly (1) in combination with a phenol/chloroform extraction and ethanol precipitation and *(iii)* the QIAprep® procedure (Qiagen, Chatsworth, CA, USA), which combines alkaline lysis of the samples with an adsorption of plasmid DNA onto a silica matrix. All procedures were originally designed for plasmid preparations from *Escherichia coli*.

Boiling method (4). Cell pellets were resuspended in 300 µL STET solution (8% sucrose, 5% Triton® X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0 and 600 µg/mL lysozyme). After incubation for 5 min on ice, the tubes were placed in a boiling water bath for 2 min. Cell debris and genomic DNA were removed by centrifugation (5 min at 10 000× *g*), and low-molecular-weight DNA was precipitated from the supernatant by adding an equal volume of isopropanol and a 30-min incubation at -20°C.

Alkaline lysis (1). Cell pellets were resuspended in 250 µL GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA). After an incubation period of 5 min at room temperature, the cells were lysed by adding 200 µL lysis buffer (200 mM NaOH, 1% sodium dodecyl sulfate [SDS]). Samples were mixed and placed on ice for 5 min. The solution was neutralized by adding 150 µL potassium acetate (KOAc) solution (5 M KOAc, pH 4.8). Cell debris and chromosomal DNA were subsequently removed from the solution by centrifugation (5 min at 10 000× *g*). Episomal DNA was further purified by phenol/chloroform extraction and ethanol precipitation.

QIAprep® Spin Plasmid Kit. This protocol uses a modified version of the previously described alkaline lysis method. Instead of performing a phenol/chloroform extraction, the lysate was loaded onto the QIAprep Spin Columns and centrifuged for 1 min. Subsequently, the columns were washed to remove residual endonucleases and salts. The DNA was eluted from the columns with 100 µL H_2O (preheated at 75°C) by centrifugation for 1 min at 10 000× *g*.

Since the primers that were used to quantify the episomal DNA can also generate amplification products from the endogenous cytokine genes, leading eventually to an overestimation of the plasmid copy number, it was important to start from pure (as opposed to genomic DNA-contaminated) DNA preparations. To check whether the DNA samples were free of residual genomic DNA, control polymerase chain reactions (PCRs) were performed. The results are depicted in Figure 1. While the QIAprep samples (lanes 1 and 6) show only one distinct band of the right size (262 bp for *IL-2* and 290 bp for *IFN-g*), the other samples gave rise to additional nonspecific amplification products (Figure 1A). To see whether these additional bands were caused by contaminating genomic DNA, we checked the episomal DNA preparations for genomic DNA by amplifying exon 2 of the phenylalanine hydroxylase (*PAH*) gene, using primers located in introns 1 and 2 (Figure 1B). While the boiling and alkaline lysis samples (lanes 2 and 3) gave rise to an amplification product of 304 bp, the QIAprep sample (lane 1) was negative. From these data, we can conclude that the boiling method and alkaline lysis based on the method of Birnboim and Doly in combination with a phenol/chloroform extraction and ethanol precipitation resulted in DNA preparations that contain residual genomic DNA. The QIAprep procedure, on the other hand, which combines alkaline lysis of the samples with an adsorption of low-molecular-weight DNA onto a silica matrix, yielded pure episomal DNA free from genomic DNA.

The episomal DNA in the different samples was quantified by PCR using the QPCR® System 5000 (PE Applied Biosystems, Foster City, CA, USA). This method involves the amplification of the target sequence with a forward primer that is biotinylated at the 5′ end and a reverse primer that is 5′-labeled with the electrochemiluminescent label Tris (2,2′-bipiridine) ruthenium (II) chelate (TBR). PCR-amplified products are captured onto streptavidin-coated magnetic beads and loaded into the electrochemical reaction chamber of the QPCR System 5000, where the beads are immobilized on an electrode

by a magnetic field. Electrochemiluminescence is produced when the sample is subjected to a voltage ramp that causes a dual oxidation of the TBR label and a co-reactant, tripropylamine (TPA). The highly unstable TPA intermediate then excites the oxidized TBR, which subsequently relaxes to the ground state by the emission of light photons at 620 nm. The amount of light emitted is directly proportional to the quantity of TBR-labeled product (2,8).

Standard samples were generated prior to DNA extraction by adding 10^{10} copies of vector DNA to 5×10^6 untransfected T lymphocytes (2000 copies/cell). As a negative control for DNA preparation and PCR, untransfected cells were used. Using an external standard, a hybridization step could be avoided, making the quantification technique faster and easier to perform.

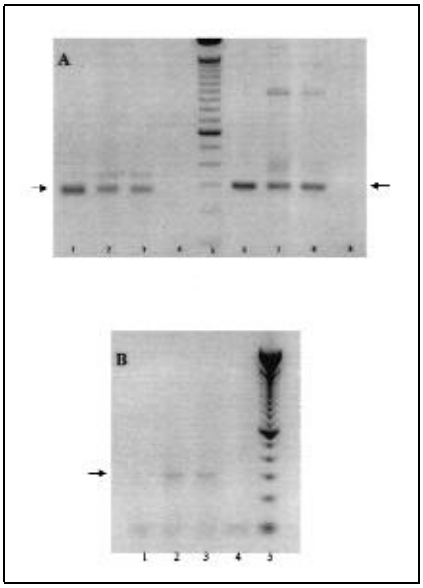

Figure 1. (A) Agarose gel electrophoresis of the PCR-amplified cytokine genes. Lanes 1–4: amplification of *IL-2*. Lane 1: QIAprep DNA; lane 2: boiling-prep DNA; lane 3: alkaline lysis DNA; and lane 4: QIAprep DNA from untransfected cells. Lanes 6–9: amplification of *IFN-g*. Lane 6: QIAprep DNA; lane 7: boiling-prep DNA; lane 8: alkaline lysis DNA; and lane 9: QIAprep DNA from untransfected Jurkat cells. Lane 5: 100-bp DNA molecular weight marker. (B) Agarose gel electrophoresis of a PCR-amplified genomic *PAH* sequence (304 bp). Lane 1: QIAprep DNA; lane 2: boiling-prep DNA; lane 3: alkaline lysis DNA; and lane 4: QIAprep DNA from untransfected cells. Lane 5: 100-bp DNA molecular weight marker. Amplification primers: PKU2 forward (5′-GAGGTTTAACAGGAATGAATTGCT-3′) and PKU2 reverse (5′-STCCTGTGTTCTTTTCATTGC-3′).

Throughout the extraction and quantification procedures, transfectant and standard samples were handled in parallel to minimize inter-sample variations. Because templates in standard and transfectant samples and the reaction environment are identical, there will be no difference in amplification kinetics. For the amplification reactions, we used the GeneAmp® PCR System 9600 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA), a calibrated second-generation thermal cycler, to eliminate possible well-to-well variations. To generate the standard curves, serial dilutions were made from the standard DNA samples, ranging from 200 to 0.4 copies per cell for the *IL-2* standard and from 100 to 0.2 copies for the *IFN-g* standard. For the transfectant samples, 3 different dilutions were used for DNA quantification. All reactions were performed in triplicate.

PCRs were carried out in 50-µL mixtures containing 5 µL template, 5 µL 10× *Taq* DNA polymerase buffer (100 mM Tris-HCl, pH 8.4, 100 mM KCl and 0.1% gelatin; Perkin-Elmer), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Pharmacia Biotech, Piscataway, NJ, USA), 5 pmol of TBR-labeled antisense primer (IL-2: 5′-ATGGTTGCTGTCTCATCAGC-3′, IFN-γ: 5′-ATGCTCTTCGACCTCGAAAC-3′), 5 pmol of biotin-labeled sense primer (IL-2: 5′-GTCACAAACAGTGCACCTAC-3′, IFN-γ: 5′-GCAGAGCCAAATTGTCTCCT-3′) and 2 U AmpliTaq® DNA polymerase (Perkin-Elmer). The amplification reactions were performed for 22 cycles (20 s at 94°C, 20 s at 55°C and 40 s at 72°C). Standard and transfectant DNA was amplified simultaneously, using the same master mixture of reagents to minimize tube-to-tube variations. To avoid contamination, all preamplification steps were carried out under a laminar flow hood. Prior to quantification, the identity of all amplified products was confirmed by comparison with the correct size based on the known length of the DNA sequence on an agarose gel.

The quantification procedure was described previously in detail (2,5–9). Briefly, 35 µL of 1× PCR buffer (10 mM Tris-HCl, pH 8.4, 10 mM KCl and 0.1% gelatin; Perkin-Elmer), and 15 µL (2 mg/mL) of streptavidin-coated

Dynabeads® (PE Applied Biosystems) were added to 5 µL of the PCR product in a plastic QPCR sample tube (PE Applied Biosystems) and shaken for 15 min at room temperature. Subsequently, 345 µL QPCR Assay Buffer (PE Applied Biosystems) containing the coreactant TPA were added, and after shaking the reaction tubes for another 30 min, the electrochemiluminescence signal was measured.

Standard curves were generated by plotting the mean luminescence signal from three measurements versus standard plasmid DNA concentrations. The episomal copy numbers in the different test samples could then be calculated by interpolation from the standard curve and are shown in Figure 2.

While conventional methods for low-molecular-weight DNA isolation and purification from eukaryotic cells are laborious and involve the use of hazardous reagents, the procedure described here is at least equivalent in terms of yield and quality of the isolated DNA and takes less than 1 h. The quantification procedure offers a convenient new possibility to determine DNA copy numbers. The accuracy and sensitivity of the automated detection system is at least comparable with previously described techniques. Combining the QIAprep DNA isolation and purification procedure with the QPCR System 5000 results in considerable reductions in both the time and the trouble required to perform this assay; quantitative data can be obtained in less than a day. These techniques can be used for a broad array of applications that involve the isolation and/or quantification of extrachromosomal DNA. For the assessment of transient transfection efficiency however, one should take into account that part of the isolated low-molecular-weight DNA is cytoplasmic and hence not active. In this case, cytoplasmic DNA can be distinguished from newly replicating episomal DNA by digesting the extracted DNA with a methylation-sensitive restriction enzyme like *Dpn*I.

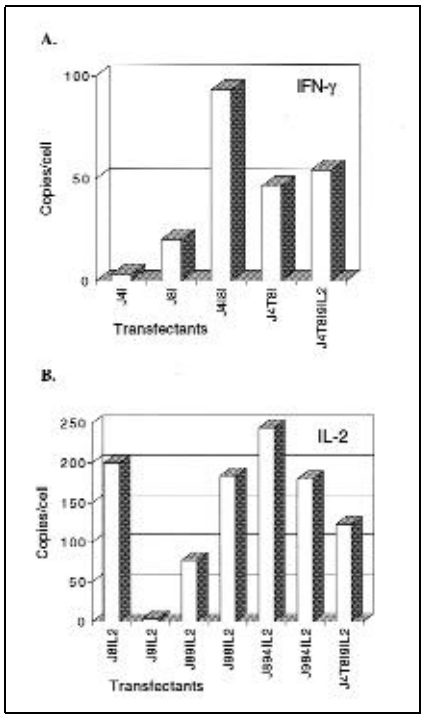

Figure 2. DNA copy number per cell. Jurkat cells were transfected with transfected cDNA constructs encoding IL-2 and IFN-γ. Approximately 8 weeks after transfection, samples were taken from selected cell lines, and vector DNA was isolated and quantitated. For each sample, three different dilutions (1/1, 1/2 and 1/5) were quantified. The average of these measurements is indicated in the figure. (A) *IFN-g* gene copies per cell. (B) *IL-2* gene copies per cell.

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