Preface

The discovery of Epstein-Barr virus (EBV) by Epstein, Achong, and Barr, reported in 1964 (*Lancet* **1**:702–703), was stimulated by Denis Burkitt's recognition of a novel African childhood lymphoma and his postulation that an infectious agent was involved in the tumor's etiology (*Nature* **194:**232–234, 1962). Since then, molecular and cellular biological and computational technologies have progressed by leaps and bounds. The advent of recombinant DNA technology opened the possibilities of genetic research more than most would have realized. Not only have the molecular tools permitted the analyses of viral mechanisms, but, importantly, they have formed the basis for discerning viral presence and, subsequently, viral involvement in an increasing number of diseases. Though in every field of science the search for further knowledge is likely to be a limitless phenomenon, the distinct goal in EBV research, namely, to gain sufficient insight into the viral–host interaction to be able to intercept the pathogenic process, is beginning to be realized.

Epstein-Barr virus research has effectively entered the postgenomic era that began with the sequencing of the first strains, cloned in the mid to late 1980s. Owing to the lack of a productive lytic system, for many years the difficulty in manipulating the viral genome virtually surpassed that of manipulating the mammalian genome. These difficulties have now largely been resolved and the use of recombinant and mini viral genomes demonstrate the continuing power of mutant analysis. Though a wealth of information on viral action has been amassed over the years, this has nevertheless been predictably dwarfed by the new questions it is possible to pose. This is evidenced by the number of laboratories working on EBV and reflected in the success of the International Association for Research on EBV and Associated Diseases and its biennial meetings (http://www.med.ic.ac.uk/ebv/home.htm). Information concerning viral infection, latency, immunogenicity, and immune evasion are being integrated into a holistic understanding of viral pathogenesis. Moreover, the decades of research on EBV provide by example a fast track for research work on newly identified, related viruses such as Kaposi's sarcoma–associated herpesvirus/human herpesvirus 8 (HHV8).

Seminal molecular techniques have opened avenues of research and several, such as Southern blotting, first described in 1975 (detailed in many variant forms in this volume), continue to yield highly informative data. With the advent of the polymerase chain reaction method in the late 1980s, assay sensitivity and detection of nucleic acids are no longer barriers to study, and new applications continue to emerge. The ability to harness homologous recombination and select for the desired products, which initiated the continuing explosion in analyzing genetic function in higher organisms by virtue of gene deletion or manipulation, has also been applied to EBV. Recently, sophisticated techniques for genomic, transcript, and proteomic comparative analyses are blooming, for which high-quality sample preparation is a prerequisite. Though these applications are not necessarily described herein, the underlying protocols for sample preparation are covered in several chapters.

All the protocols an EBV researcher could desire cannot possibly be covered in one single volume; however, we have endeavored to include many of the principal methods used and described by experts. Moreover, most of these protocols can be applied directly, or easily adapted, to address questions in fields of molecular biological research unrelated to EBV studies.

The editors would like to take this opportunity to thank John Walker, the series editor, and all the staff at Humana Press who were involved in producing this volume.

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Analysis of Replication of oriP-Based Plasmids by Quantitative, Competitive PCR

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1. Introduction

The quantitative, competitive polymerase chain reaction (PCR) assay outlined in this chapter was designed for the detection and quantitation of replicated DNAs in both short-term and long-term assays ***(1)***. Quantitative, competitive PCR can be used to study both the contribution of proteins to the replication of oriP-based plasmids ***(1)*** as well as the requirements for specific DNA sequences to support replication of a plasmid ***(2)***. Advantages of this assay include an increased sensitivity and a decreased time required to analyze samples relative to DNA blots, the traditional assay used to study replication of oriP-containing plasmids in the presence of EBNA-1 ***(3–8)***.

In long-term experiments, quantitative, competitive PCR can be used to determine whether replicated DNAs are maintained as plasmids in cells under selection and to determine how many copies of those plasmids are present in those cells. However, this assay does not allow the determination of what type of rearrangement, if any, the input DNA may have undergone to be maintained as a plasmid in the host cells under drug selection. Instead, DNA blots are more useful to determine the nature of rearrangements that may occur in the input DNAs. Therefore, although quantitative, competitive PCR does have limitations, it is a sensitive and powerful experimental approach for studying the effects of proteins and the requirements for DNA sequences involved in replication.

For the quantitative, competitive PCR assay, primers are chosen that allow simultaneous amplification of up to three templates: the reporter DNA, the replication-defective DNA and the competitor DNA. Both the reporter DNA, and the replication defective DNA (generated in a *dam* + strain of *Escherichia coli* to incorporate the prokaryotic methylation signature) are introduced into the host cell. Subsequently, after culture, low molecular weight DNA is harvested from cells using a modified Hirt method ***(9)*** and digested with *Dpn*I to fragment any remaining DNA with a prokary-

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otic methylation pattern, which corresponds to the unreplicated, input DNA. The reporter DNA will be amplified during the PCR only if it has been replicated by the host cell and is therefore *Dpn*I-resistant. The replication defective DNA serves as an internal control and will be amplified during the PCR only if it too has been replicated, or if the *Dpn*I digestions have not gone to completion. Mammalian cells do support synthesis of prokaryotic vectors inefficiently for short times ***(1,2,10)***.

In order to quantitate the PCR products, known concentrations of the competitor DNA are added to a series of PCR assays. The use of ^{32}P-end labeled oligonucleotide primers allows incorporation of ^{32}P into the PCR products and facilitates the quantification. In a given PCR, the competitor DNA and the reporter DNA will be amplified with equal efficiency when the two templates are present in equal amounts, consequently, the amount of radioactive label incorporated for each template will be identical.

Oligonucleotide primers for use in competitive, quantitative PCR should be designed that will amplify a region of DNA on the reporter plasmid, the replication-defective plasmid and the competitor plasmid simultaneously. This region of DNA should be designed so that it varies in length between the three plasmids, so that amplification will yield products of three distinguishable sizes. For example, in previously reported experiments ***(1)*** the replication-competent reporter plasmid, *oriP-Bam*HI C-Luc, contains a wild-type gene encoding aminoglycoside phosphotransferase II. The replication-defective plasmid, *oriP*-minus (that serves as an internal control for digestion of nonreplicated DNA by *Dpn*I), lacks oriP and contains a 233 bp insertion at the *Msc*I site within the gene encoding aminoglycoside phosphotransferase II. The competitor DNA introduced into the PCR assay lacks oriP and contains a 222 bp deletion between the *Bsa*A1 and *Msc*I sites within the gene encoding aminoglycoside phosphotransferase II. One pair of primers will anneal to all three plasmids and amplify the corresponding fragment from each plasmid with equal efficiency. The sizes of amplified fragments generated for each of these constructs using one set of primers are 964, 742, and 1197 bp, respectively. Other primers and templates for this assay can be readily designed and used to monitor replication of oriP-based plasmids. However, these templates must be tested for their ability to be amplified with equal efficiency using the corresponding primers.

2. Materials

2.1. Cell Culture and DNA Transfection

1. Appropriate complete tissue culture medium (TCM): For example, for 143B cells ***(11)***, Dulbecco's Modified Eagle's medium (DMEM-HG), 10% calf serum, 0.2 mg/mL streptomycin sulfate, 200 U/mL penicillin G potassium. Store at 4°C.
2. Phosphate buffered saline (PBS): 0.137 *M* NaCl, 2.7 m*M* KCl, 5.4 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4. Adjust pH to 7.4 and filter through a 0.2-µm filter.
3. 1X trypsin: Dilute 10X trypsin (Gibco BRL, containing 0.5% trypsin, 5.3 m*M* ethylenidiaminetetraacetic acid (EDTA)-4Na) in PBS. Filter through a 0.2-µm filter and store at 4°C.
4. 1X Eosin Y: 0.1% Eosin Y, 0.2% sodium azide in PBS. Filter through a 0.2-µm filter.
5. TCM-H: Add 1/20 vol of 1 *M* HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Gibco BRL cat no 15630–023 or equivalent), pH 7.4–7.6, to complete tissue culture medium, giving a final concentration of 50 m*M* HEPES. Store at 4°C.

6. Tissue culture flasks and dishes.
7. 50-mL conical tubes.
8. Hemocytometer.
9. 37°C CO_2 humidified incubator.
10. CsCl-gradient purified plasmid DNAs.

2.2. Isolation of Low Molecular Weight DNA

1. Cell resuspension buffer: 50 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA, 0.1 *M* NaCl.
2. Lysis buffer: 1.2% sodium dodecyl sulfate (SDS), 10 m*M* EDTA, 0.2 *M* Tris-HCl, pH 7.6.
3. 5 *M* NaCl.
4. RNAse A: 20 mg/mL, heat to >70°C for 20 min, store at –20°C.
5. Proteinase K: 20 mg/mL, store at –20°C.
6. Phenol:chloroform: 1:1 ratio, buffered to pH 8.0.
7. Chloroform.
8. Glycogen (20 mg/mL).
9. 100% ethanol.
10. 5 *M* ammonium acetate.
11. 70% ethanol in H_2O.
12. 1XTE7.5: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA.
13. 0.1X TE7.5: 10-fold dilution of 1X TE7.5 with H_2O.
14. Microfuge tubes and microcentrifuge.

2.3. Competitive, Quantitative PCR

2.3.1. Digestion of Sample DNA and Competitor DNA

1. *Dpn*I and other restriction enzymes and their buffers: store at –20°C.
2. 10X KGB: 1 *M* K-glutamate, 250 m*M* Tris-acetate, 100 m*M* Mg-acetate, 5 m*M* -mercapto-ethanol, 0.5 mg/mL bovine serum albumin (BSA); Store at –20°C.
3. Phenol:chloroform: 1:1 ration, buffered to pH 8.0.
4. Chloroform.
5. 100% ethanol.
6. 5 *M* ammonium acetate.
7. 70% ethanol in H_2O.
8. 1XTE7.5 (as in **Subheading 2.2.**).
9. 0.1X TE7.5: 10 fold dilution of 1X TE7.5 with H_2O.
10. Microfuge tubes and microcentrifuge.

2.3.2. Agarose Gel Elecrophoresis

1. 1X TBE: 90 m*M* Tris, 80 m*M* boric acid, 2 m*M* EDTA.
2. 1.0% or 1.5% agarose (as indicated) in 1X TBE, microwaved to melt.
3. 5X Blue Juice: 0.05% Bromophenol Blue, 30% glycerol in H_2O.
4. Ethidium bromide (10 mg/mL).
5. Electrophoresis apparatus for slab agarose gels.
6. UV light transiluminator.

2.3.3. End-Labeling Primers

1. T4 polynucleotide kinase (New England Biolabs), store at –20°C.
2. 10X T4 polynucleotide kinase buffer (New England Biolabs), store at –20°C.
3. ^{32}P -ATP (6000 Ci/mmol), e.g., Dupont NEN, store at –20°C.

4. QIAquick nucleotide removal kit (Qiagen) or equivalent kit.
5. 2-mL microfuge tubes.
6. 10 m*M* Tris-HCl, pH 8.0, heat to 60°C prior to use.

2.3.4. Competitive, Quantitative PCR

1. Microfuge tubes and microcentrifuge.
2. 0.1X TE7.5 (as in **Subheading 2.3.1.**).
3. 10X Taq buffer (Boehringer), store at –20°C.
4. 20 µ*M* dNTPs, store at –20°C.
5. 20 µ*M* 5' primer, store at –20°C.
6. 20 µ*M* 3' primer, store at –20°C.
7. Taq polymerase (5 U/µL, Boehringer), Store at –20°C.
8. 500 µL GeneAmp tubes (Perkin Elmer) or equivalent.
9. PCR thermocycler (e.g., Perkin Elmer thermocycler 480).
10. Mineral oil.
11. India ink.
12. 7.5% trichloroacetic acid (TCA), in H_2O.
13. Whatman 3MM paper.
14. DE81 paper (Fisher 05-717-A).
15. Saran wrap.
16. Vacuum gel dryer.
17. PhosphorImager with screens (e.g., Molecular Dynamics).

3. Methods

3.1. DNA Transfection into Cells and Subsequent Isolation of Low Molecular Weight DNA

1. Harvest cells and count viable cells as described in Chapter 12, **Subheading 3.1.**, **step 1** and resuspend viable cells to 2×10^7 cells/mL in TCM-H.
2. Electroporate 10 µg of each plasmid DNA (oriP-containing DNA [the reporter], oriP-minus DNA [replication defective] and effector DNA encoding a derivative of EBNA-1) into 0.5 mL of cell suspension (1×10^7 cells) as described in Chapter 12 (*see* **Note 1**).
3. Incubate one electroporated sample in 20 mL of complete tissue culture medium in a 15-cm dish at 37°C in 6% CO_2 for 94–98 h.
4. Harvest and count the viable cells as described in Chapter 12, **Subheading 3.1.**, **steps 1–4**.
5. Resuspend the cells to 2×10^7 cells/mL in cell resuspension buffer and transfer the cells to microfuge tubes, filling the tubes to no more than one third (*see* **Note 2**).
6. To each sample, add an equal volume of lysis buffer. Rock gently for 10 min at room temperature. (Do not pipet or vortex the sample as this may shear the chromosomal DNA).
7. Add 0.5 mL of 5 *M* NaCl/1 mL sample (equivalent to 2×10^7 cells). Rock gently for 10 min at room temperature.
8. Incubate the samples at 4°C for 24–48 h to precipitate the high molecular weight DNA.
9. Pellet the high molecular weight DNA and cell debris by centrifugation at 9600*g* at 4°C for 45 min in a microfuge or other appropriate centrifuge (*see* **Note 2**).
10. Carefully transfer the supernatant, which contains the majority of the low molecular weight DNA to a fresh tube. Discard the pellet (*see* **Note 3**).
11. Incubate the supernatant for 2 hours at 42°C with RNAse A added to a final concentration of 0.1 mg/mL.

12. Incubate the supernatant for 2 h at 42°C with Proteinase K added to a final concentration of 0.2 mg/mL.
13. Extract the supernatant with an equal volume of phenol:chloroform, then an equal volume of chloroform by vortexing the sample with the organic solvent, centrifugation (10,000–16,000*g* for 30 s), and collection of the aqueous phase. Repeat if the samples contain a large amount of proteinaceous material.
14. Add 1 µL of glycogen (20 mg/mL) as carrier to each sample for precipitation. Add ammonium acetate to each sample to a final concentration of 0.3 *M*. Add 2 vol of 100% ethanol and mix well. Incubate the samples on dry ice for approx 20 min, or at –70°C for approx 1 h or at –20°C overnight, to precipitate the DNA.
15. Pellet the DNA by centrifugation at 9600*g* for 10 min in a microfuge, decant the ethanol. Wash the pellet with 70% ethanol and centrifuge at 9600*g* for 5 min, decant the ethanol. Dry the low molecular weight DNA in a Speed Vac or air dry.
16. Resuspend the samples in 300–500 µL of 1X TE7.5. Store the samples at –20°C.

3.2. Competitive, Quantitative PCR

3.2.1. Digestion of Low Molecular Weight Sample DNA

1. Incubate the samples overnight at 37°C with 100–160 U of *Dpn*I per 1×10^7 cell equivalents of low molecular weight DNA (in the appropriate buffer) to digest any unreplicated DNA.
2. Add a further 80 U of *Dpn*I and 20–40 U of an appropriate restriction enzyme (to linearize the input plasmid DNA) and incubate at 37°C for one hour.
3. To determine whether the digestions with *Dpn*I have gone to completion, perform one nonradioactive, competitive PCR per sample using 0.1 pg or less of competitor DNA in the reaction (*see* **Subheading 3.2.4.**).
4. If the digestions have gone to completion, extract the sample with an equal volume of phenol:chloroform and then an equal volume of chloroform as described in **Subheading 3.1.**, **step 13**.
5. Precipitate the DNAs as described in **Subheading 3.1.**, **steps 14–15**.
6. Resuspend the samples in 0.1X TE7.5 to a concentration of 1×10^5 cell equivalents/µL. Store the samples at –20°C (*see* **Note 4**).

3.2.2. Generation of Competitor DNA

1. Linearize 20 µg of competitor plasmid DNA with an appropriate restriction enzyme. To check that the digestion is complete, take an aliquot of competitor DNA and add loading buffer (to 1X Blue Juice). Electrophorese the aliquot in a 1.0% agarose gel (containing approx 0.5 µg/mL ethidium bromide) in 1X TBE.
2. Purify the competitor DNA sample by extraction with an equal volume of phenol:chloroform and then an equal volume of chloroform (*see* **Subheading 3.1.**, **step 13**).
3. Precipitate the competitor DNA as described in **Subheading 3.1.**, **steps 14–15**.
4. Resuspend the competitor DNA to an estimated concentration of 0.5–1 mg/mL in TE7.5.
5. Determine the concentration of the competitor DNA using a Hoescht dye assay ***(12)***, or by comparing the intensity under ultraviolet (UV) light of serial dilutions of agarose gel electrophoresed competitor DNA in the presence of ethidium bromide (0.5 µg/mL) to similar dilutions of known concentrations of a standard DNA ***(13)***.
6. Generate a working stock of competitor DNA at 1–10 ng/mL in 1XTE7.5 for PCR. Store at –20°C in a screw cap tube in a nonfrost free freezer.

3.2.3. End-Labeling Primers

1. Incubate (separately) 75 pmol of each primer with 10 U of T4 polynucleotide kinase, and 125 pmol of ^{32}P -ATP (750 mCi) in 1X T4 polynucleotide kinase buffer in a 30 µL total reaction volume for 30 min at 30°C (*see* **Note 5**).
2. Add an additional 10 U of T4 polynucleotide kinase to each primer reaction and incubate for an additional 30 min at 30°C.
3. Separate the primers from unincorporated ^{32}P -ATP by purification using the QIAquick nucleotide removal kit according to manufacturer's instructions as outlined below in **steps 4–8**.
4. Add 300 µL of buffer PN (Qiagen kit) to the ^{32}P-end labeled primer and mix. Incubate for 1 min at room temperature. Transfer the sample mix to a QIAquick column placed in a 2 mL microfuge tube. Centrifuge the column at approx 4000*g* in a microfuge for 1 min. Discard the radioactive eluate.
5. Transfer the column to new microfuge tube. Add 300 mL of buffer PE (Qiagen kit) to the column. Centrifuge the column at approx 4000*g* in a microfuge for 1 min. Transfer the column to a new microfuge tube and discard the radioactive eluate.
6. Add 400 µL of buffer PE (Qiagen kit) to the column. Centrifuge the column at approx 4000*g* in a microfuge for 1 min. Transfer the column to a new microfuge tube and discard the radioactive eluate.
7. Centrifuge the column at approx 9600*g* in a microfuge for 30 s. Transfer the column to a new microfuge tube and discard the radioactive eluate.
8. Add 40 µL of 10 m*M* Tris, pH 8.0 (preheated to 60°C) to the column. Centrifuge the column at approx 9600*g* for 1 min to elute the ^{32}P-end labeled primer. Transfer the ^{32}P-labeled primer to a screw-cap tube. Store at –20°C.

3.2.4. Quantitative, Competitive PCR Assay

1. Generate the following master reaction mix containing a multiple of each reagent to equal the number of samples to be analyzed plus two extra (*see* **Note 6**). Vortex to mix. Master reaction mix (per one PCR) for a total reaction volume of 100 mL: 10 µL 10X Taq buffer (Boehringer), 1 µL 20 m*M* dNTPs, 1 µL 20 µ*M* 5' primer, 1 µL 20 µ*M* 3' primer, 0.1 µL ^{32}P -ATP-labeled 5' primer, 0.1 µL ^{32}P -ATP-labeled 3' primer, 75.3 mL H_2O, 0.5 µL 5 U/µL Taq polymerase (Boehringer).
2. Dilute the competitor DNA from the working stock (**Subheading 3.2.2.**) to known concentrations (e.g., 0.00025, 0.0010, 0.0040, 0.16, 0.64, and 2.5 pg/µL) in 0.1X TE7.5 in order to generate a standard curve (*see* **Note 7**).
3. To 500 µL tubes add 10 µL of one concentration of competitor DNA, and 1 µL of sample DNA (1×10^5 cell equivalents/µL). Set up five reactions per sample, with an increasing amount of competitor DNA per reaction. Then aliquot 89 µL of the master reaction mix to each tube and mix to give a total volume of 100 µL/tube. Overlay with 70 µL of mineral oil.
4. Set up the PCR using the following conditions: initially denature the DNA templates at 94°C for 5 min once. Then set cycle: denature at 94°C for 30 s, anneal at 55°C for 30 s, and elongate at 72°C for 1 min. Repeat for 20-25 cycles. Finally, elongate at 72°C for 10 min, and transfer samples to 4°C. Store the samples at –20°C.
5. Take 15 µL of each PCR reaction, add 4 µL of 5X Blue Juice, and electrophorese the samples in a 1.5% agarose gel (containing approx 0.5 µg/mL ethidium bromide) in 1X TBE. When loading the gel, do not load the lane between the lowest amount of competitor of one sample and the highest amount of competitor in the next sample in order to avoid obscuring the signal of the lowest amount of competitor DNA and therefore compromising data analysis. Electrophorese overnight at 0.5–1 V/cm or for approx 4 h at 4 V/cm.

6. Examine the gel under UV light and mark the location of the molecular weight markers with a needle dipped in India Ink.
7. Precipitate the DNA in the gel by incubating the gel in 7.5% TCA for approx 30 min, until the dye front is yellow.
8. Optional: Place the gel on a stack of dry Whatman 3MM paper, cover with Saran wrap and place a book or equivalently weighted flat object on top for 15 min. This will facilitate wicking excess buffer out of the fixed gel, and reduce the amount of time required to dry the gel.
9. Transfer the gel onto a sheet of DE81 paper with two new sheets of Whatman 3MM paper underneath. Cover the gel with Saran wrap. Dry the gel in a gel dryer for 2–2.5 h. Remove the dried gel from the gel dryer and replace the Saran wrap with a new sheet.
10. Expose the dried gel to a PhosphorImager screen overnight. Collect data from the PhosphorImage (*see* **Fig. 1A**) and analyze as described in **Subheading 3.2.5.**

3.2.5. Data Analysis

1. To measure the amount of replicated reporter DNA in the sample, plot the graph log (molecules of competitor DNA) vs log (PhosphorImager Units of competitor DNA/PhosphorImager Units of reporter DNA) (*see* **Fig. 1B**).
2. When the amount of the competitor DNA is equivalent to the amount of the reporter DNA in the sample, the two templates will be amplified with equal efficiency. Graphically, this represents the point on the x-axis where the log of 1/1 = 0. Therefore, the inverse log of the intercept equals the number of *Dpn*I-resistant molecules present in 1×10^5 cell equivalents of the sample, assuming all cells took up DNA upon transfection.
3. The average number of replicated molecules per transfected cell can be determined by correcting for the transfection efficiency of the cell line used in the experiment. To do this, divide the number of *Dpn*I-resistant molecules present in 1×10^5 cell equivalents of the sample by 1×10^5 cells and multiply that number by the transfection efficiency of the cell line tested. A method for determining the transfection efficiency of cell lines is described in Chapter 12.
4. To ensure that the *Dpn*I digestions have gone to completion, plot the graph log (molecules of competitor DNA) vs log (PhosphorImager Units of competitor DNA/PhosphorImager Units of *oriP*-minus DNA) and analyze the data as described earlier.

4. Notes

1. DNA may also be introduced by other means (e.g., calcium phosphate precipitation ***[14]***) depending on the cell type.
2. Use larger tubes appropriate to the centrifugation in **Subheading 3.1., step 9** for larger sample volumes.
3. If desired, the high molecular weight, chromosomal DNA separated from the low molecular weight DNA can be analyzed as well. To do so, resuspend the pellet (containing primarily chromosomal DNA and cell membranes) in 1 m*M* EDTA, 0.1 *M* NaOH. This resuspension takes time and can be accelerated by incubating at 45°C and by gentle vortexing. Once resuspended, extract with phenol, phenol:chloroform, and chloroform. Precipitate the sample as described in **Subheading 3.1.**, **steps 14–15**. However, do not dry the sample. Instead, immediately resuspend the high molecular weight DNA in 1X TE7.5. Continue to process high molecular weight DNA as described in **Subheading 3.1.**, **steps 11–16**.

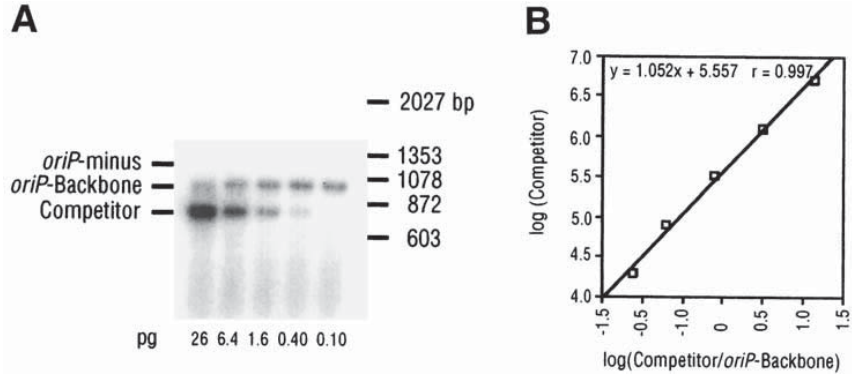

Fig. 1. Short-term replication of a reporter plasmid containing oriP in 143 cells that stably express wild-type EBNA-1 as measured by quantitative, competitive PCR. **(A)** Example of PhosphorImage of a sample analyzed by quantitative, competitive PCR. Ten µg each of reporter DNA (*oriP*-backbone) ***(2)*** and replication-defective DNA (*oriP*-minus) ***(1)*** were introduced into 1×10^7 143/EBNA-1 cells ***(15)*** and analyzed as described in this chapter. Briefly, the low molecular weight DNA was harvested 12 d postelectroporation by Hirt extraction ***(9)***, digested with *Dpn*I to fragment any unreplicated DNA, and *Acc*I to linearize the templates. Five quantitative, competitive PCRs with varying amounts of competitor DNA ***(1)*** were performed for the sample. 15 µL of each PCR were run on a 1.5% agarose gel in 1X TBE, and data were analyzed using a PhosphorImager. The migration patterns of molecular weight markers are noted to the right of the gel and the migration patterns of *oriP*-minus, o*oriP*-backbone, and competitor DNAs are shown to the left. The amount of competitor DNA in pg added to each PCR is noted below each lane. **(B)** Graph of the data from the sample shown in (A). Data from the PhosphorImage were analyzed as described in this chapter. Briefly, to measure the amount of replicated reporter DNA (in this case, *oriP*-backbone) in the sample, the graph log (molecules competitor) vs log (PhosphorImager units competitor/PhosphorImager units reporter) was plotted. r = correlation coefficient. The number of *Dpn*I-resistant molecules per 1×10^5 cell equivalents of sample was determined from the inverse log of the intercept. The number of replicated molecules per transfected cell was determined by dividing by the number of cell equivalents used in the competitive, quantitative PCR and multiplying by the transfection efficiency (approx 25% under the conditions used in this example) of the cell line. In this example, approx 14 copies of replicated *oriP*-backbone was present per transfected cell.

4. Prior to setting up a large experiment using radiolabeled primers, it is often useful to run a subset of samples in a nonradioactive competitive, quantitative PCR (*see* **Subheading 3.2.4.**) to ensure that the number of replicated plasmids detected are within the chosen range of concentrations of competitor DNA.
5. To increase the efficiency of the labelling reaction, the reaction samples can be incubated on ice overnight instead of 30 min at 30°C.
6. The quantitative, competitive PCR assay is linear over the range of at least 1×10^4–1×10^6 cell equivalents of sample, and between at least 0.025 and 26 pg of competitor DNA. If

1×10^4 cell equivalents of sample DNA are used in the PCR reaction, 1×10^5 cell equivalents of a low molecular weight DNA extracted from non-transfected cells, processed as described earlier, should be added to each PCR as a carrier DNA. If 1×10^6 cell equivalents of sample are used per point of standard curve, low molecular weight DNA from at least 5×10^6 cells must be harvested initially. The lower limit of detection in the assay can be adjusted by either starting with more sample DNA in each PCR or using less Competitor DNA and increasing the number of cycles used during the PCR analysis.

7. A new standard curve (even from the same competitor DNA stock) must be generated for each experiment.

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