Quantitation of Rare DNAs by PCR

This unit presents a protocol that uses the polymerase chain reaction (PCR) to quantitate the numbers of a particular DNA sequence from 1 to 20,000 molecules per sample. In addition, it helps assess the presence of contaminating sequences, the bane of this kind of procedure.

The DNA of interest is prepared and its concentration is determined. A known amount of this DNA is then mixed with two sets of oligonucleotide primers, one set specific for the DNA of interest (e.g., a virus) and the other set specific for an internal control (e.g., a single-copy gene encoded by the host organism). The sequences between the primers are amplified, electrophoresed on a gel, transferred to a filter, and probed with oligonucleotides specific for each amplified product. The amounts of the amplified products from the DNA of interest can then be quantitated by comparison to the internal control. For simplicity, the protocol is written in terms of quantitating viral DNA molecules relative to host cellular sequences; however, it can be adapted readily for other applications.

A hallmark of the immune system is that low-frequency cellular events are selected and amplified in the early course of an immune response. Thus, it is particularly valuable to employ methods that are both exquisitely sensitive and also specific for the presence of somatically generated antibody and T cell–receptor genes. The methods for quantifying rare DNAs described in this unit can be applied to the identification of such genes and also can be used for typing specific alleles of the polymorphic genes encoded by the major histocompatibility complex (MHC).

**Materials**

- Cells or tissue sample of interest
- Proteinase digestion buffer (see recipe)
- 20 mg/ml proteinase K (store at −20°C)
- Phenol buffered with 50 mM Tris-Cl/10 mM EDTA, pH 7.4 (store at room temperature; see UNIT 10.1 for buffering procedure)
- 24:1 chloroform/isoamyl alcohol (UNIT 10.1)
- 10 M ammonium acetate
- Cold 100% ethanol
- 70% ethanol
- TE buffer, pH 7.5 (APPENDIX 2)
- Internal control DNA from uninfected cells or tissues (see Critical Parameters)
- DNA molecular weight markers (UNIT 10.4)
- Reaction mix cocktail (see recipe)
- Mineral oil
- 0.8 U/µl *Taq* DNA polymerase
- End-labeled oligonucleotide primers for hybridization (see recipe)
- Screw-cap microcentrifuge tubes, autoclaved
- Microcapillary pipets
- Densitometer (optional)

Additional reagents and equipment for tissue sample preparation (UNIT 10.2), agarose gel electrophoresis (UNIT 10.4) or ethidium bromide dot quantitation (UNIT 10.5), PCR (UNIT 10.20), Southern hybridization and UV cross-linking (UNIT 10.6A), and hybridizing blots with oligonucleotides (UNIT 10.6B)

**NOTE:** Use sterile, distilled water to prepare all reagents. Do not use diethylpyrocarbonate (DEPC) to treat reagents. To avoid contamination with unwanted nucleic acids, prepare
reagents and solutions solely for use in this protocol (see Critical Parameters and Troubleshooting). Wear disposable gloves and change them frequently.

**Prepare the DNA**

1. Place cells or tissue sample in a screw-cap microcentrifuge tube. Add ~100 µl proteinase digestion buffer per ~2 × 10^6 cells and 20 mg/ml proteinase K to 100 µg/ml. Incubate sample overnight at 50°C.

   *This is a modification of the protocol in UNIT 10.2. It is best to process samples in the order of increasing likelihood of their containing the sequences of interest. Perform the extractions away from where products of the amplification reactions (PCR products) or large quantities of plasmid DNA are handled. Wear disposable gloves and change them frequently.*

   *Always include several negative control samples that contain no viral sequences. Take care not to contaminate cells or tissue samples with unwanted DNA sequences.*

2. Mix sample gently up and down with a 200-µl microcapillary pipet. Add 100 µl buffered phenol to digestion mixture, mix gently, add 100 µl of 24:1 chloroform/isoamyl alcohol, and mix gently.

   *The initial mixing shears the DNA very slightly, allowing for more efficient extraction. The gentle extraction maintains the DNA as high-molecular-weight molecules. This aids in estimating its concentration but is less important if mere detection is desired.*

   *Screw-cap microcentrifuge tubes and microcapillary pipets minimize aerosol contamination, which commonly results from microcentrifuges and automatic pipettors. To prepare microcapillary pipets, autoclave on dry cycle or bake in a 200°C oven overnight. Dedicate a single bulb for each reagent used. Positive-displacement pipets with disposable tips and plungers are a more expensive, but easier-to-use alternative to microcapillary pipets.*

3. Microcentrifuge 5 min at high speed, room temperature, and transfer aqueous phase (which contains DNA) to a new microcentrifuge tube.

   *Even with the use of screw-cap microcentrifuge tubes, avoid using a microcentrifuge that has been used for PCR products or large amounts of plasmid DNA.*


5. Microcentrifuge 5 min at high speed, room temperature, and add aqueous phase to aqueous phase from step 3.

6. Extract aqueous phase twice with an equal volume of 24:1 chloroform/isoamyl alcohol, each time microcentrifuging 5 min at high speed, room temperature, to separate the phases.

7. Add 10 M ammonium acetate to 2.5 M (final) in the last aqueous phase and mix gently. Add 2.5 vol cold 100% ethanol and mix gently. Place on crushed dry ice 30 min. Microcentrifuge 15 min at high speed, 4°C, to pellet DNA, and pour off supernatant.

   *The use of ammonium acetate during ethanol precipitation is critical for subsequent efficient amplification. DNA precipitated from sodium acetate does not amplify as well, but can be improved by reprecipitation with ethanol from 2.5 M ammonium acetate.*

8. Wash DNA pellet with 1 ml of 70% ethanol by inverting the tube several times. Microcentrifuge 15 min, pour off supernatant, and dry pellet in a desiccator under vacuum or in a Speedvac evaporator.

   *Avoid using a desiccator or evaporator that has been used for PCR products or large amounts of plasmid DNA.*
9. Resuspend pellet in TE buffer, pH 7.5 (100 µl for a sample prepared from 2 × 10^6 cells). Store DNA at 4°C.

   The final DNA concentration should be 5 to 100 ng/µl. It may take several hours or overnight before the DNA is totally dissolved.

10. Estimate DNA concentration either by running an aliquot on an agarose gel alongside known amounts of standard DNA (UNIT 10.4) or more rapidly by ethidium bromide dot quantitation (UNIT 10.5).

   This step is important because the subsequent quantitation will be less accurate if ≥1 µg or ≤10 ng DNA is assayed. Estimating the amount of DNA on a gel has the advantage over ethidium bromide dot quantitation in that it assesses how intact the DNA is. The extra precautions outlined above will diminish yield somewhat.

**Amplify the DNA by PCR**

11. Prepare one tube containing 110 ng, one tube containing 90 ng, and several tubes each containing 100 ng DNA from uninfected cells or tissue. Use these tubes to make a set of 10-fold serial dilutions of the sequence of interest as follows: Add a known amount (e.g., 20,000 molecules) of DNA containing the sequence of interest to the tube containing 110 ng DNA and mix. Then add one-tenth of this material to a tube containing 100 ng DNA and mix. Add one-tenth of this material to a new tube containing 100 ng of DNA and mix. Repeat several more times until a tube contains ≤10 molecules of the sequence of interest. Then add one-tenth of the material from that tube to the tube containing 90 ng of DNA.

   The final total volume of each tube should be ≤71 µl.

   To compare with samples from a mouse infected with a virus, prepare the first tube so there is one copy of viral DNA per mouse cell equivalent in 100 ng of DNA from an uninfected mouse, which is ~20,000 molecules of viral DNA. Make serial dilutions to yield samples with 0.1, 0.01, 0.001 (etc.) copy per cell equivalent. As a control, prepare a sample containing 100 ng uninfected cell DNA without added viral sequences.

12. For each amplification reaction, prepare a screw-cap microcentrifuge tube containing 24 µl of reaction mix cocktail and enough sterile distilled water for a final volume of 100 µl (after addition of DNA and Taq DNA polymerase, steps 13 and 15). Mix and overlay each reaction with 2 drops (~100 µl) mineral oil, to completely cover the surface of the reaction mixture (also see UNIT 10.20).

   Overlaying with oil at this step rather than after adding Taq DNA polymerase helps minimize contamination because everything is assembled before adding the experimental samples. There is also less chance of contaminating the oil with DNA.

13. Open only those tubes that will contain equivalent samples (e.g., duplicate samples or samples from animals infected with the same virus inocula). Add 100 ng sample DNA to each appropriate tube and close the tubes. Microcentrifuge briefly to mix.

   Begin with the no-virus control (e.g., 100 ng mouse DNA) followed by other negative controls (include at least two other negative controls). Proceed in order of increasing likelihood that a sample will contain viral sequences of interest, adding serial dilution tubes last. Adding samples in this order lessens the likelihood that aerosols of more concentrated samples will contaminate less concentrated samples.

14. Heat-denature the samples 1 min at 94°C, either in a water bath or in an automated cycler.

15. Open tubes (containing equivalent samples) and add 5 µl of 0.8 U/µl Taq DNA polymerase to each tube. Close tubes and repeat steps 13 to 15 with the next set of equivalent samples.
Denaturing before adding the Taq DNA polymerase puts the polymerase through one less cycle at high temperature.

16. Microcentrifuge tubes briefly. Cycle tubes one time for 2 min at 55°C (reannealing) and 3 min at 72°C (extension).

The microcentrifuge spin mixes in the polymerase. This step completes the cycle started by the heat denaturation in step 14.

17. Run amplification program as follows:

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Time</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 cycles</td>
<td>1 min</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td>2 min</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>3 min</td>
<td>72°C</td>
</tr>
<tr>
<td>1 cycle</td>
<td>7 min</td>
<td>72°C</td>
</tr>
<tr>
<td>Final step</td>
<td></td>
<td>indefinitely</td>
</tr>
</tbody>
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Analyse the PCR products

18. Electrophorese product aliquots on an appropriate agarose (UNIT 10.4) or non-denaturating polyacrylamide gel. Include lanes with DNA molecular weight markers that will be visible both upon ethidium bromide staining and by autoradiography.

Chory and Baldwin (1994) describes a procedure for non-denaturing PAGE.

One-tenth of the reaction should be sufficient for detection of the amplified host sequences by ethidium bromide staining and for detection of the amplification products of single molecules following hybridization. Do not use markers that are so radioactive that they will obliterate other lanes during autoradiography.

19. Stain and photograph the gel.

The DNA fragment corresponding to the PCR product from the host DNA should be readily visible following ethidium bromide staining and will serve as a measure of the efficiency of each amplification reaction. Don’t be surprised to see a number of other bands, especially of higher molecular weights, which are nonspecific PCR products. However, if the reaction has been optimized (UNIT 10.20), the specific PCR product should predominate. Check for specificity by restriction endonuclease digestion (if the product contains a site; UNIT 10.8) or by hybridization with an internal oligonucleotide as described below.

20. Transfer gel to a nitrocellulose or nylon filter (UNIT 10.6A). If desired, UV-cross-link DNA to filter.

Choice of filter and transfer method is an individual preference; however, electroblotting from acrylamide gels to nylon filters followed by UV cross-linking (UNIT 10.6A) has proven to be a quantitative method for transferring and retaining small DNA fragments on filters during multiple cycles of hybridization, stripping, and reprobing.

21. Prehybridize the filter and hybridize with end-labeled oligonucleotide specific for the sequence of interest (UNIT 10.6B). Analyze by autoradiography.

If each reaction is amplified with similar efficiency (which can be assessed from the ethidium bromide-stained gel), the dilution series should give a monotonically decreasing signal of one predominant band with increasing dilution. It should be possible to detect a signal from dilutions containing only one or a few molecules. There should be no signal from the negative controls. A reasonable estimate for the number of molecules of the sequence of interest in each sample can be obtained by comparison with the dilution series, assuming that each reaction amplified with similar efficiency.

22. For more quantitative analysis, strip the filter of the previous probe by boiling in water 15 min (if necessary) and hybridize with a probe specific for the host single-copy sequence. Quantitate the signals by densitometric scanning (following the densitometer manufacturer’s instructions) and compute a standard curve from the dilution
series, normalizing to the host sequence signals. Determine the number of molecules in the experimental samples by interpolation from the standard curve.

The standard curve will ideally give a linear relationship between the log of the autoradiographic signal and the log of the amount of DNA. However, it may not necessarily be completely linear, and it is unlikely to have a slope of 1. At the high end (0.1 to 1 copy per cell equivalent), it may plateau. If this is a problem, it may be necessary to reduce the number of amplification cycles or vary other parameters (see UNIT 10.20).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 5.

**Amplification buffer, 10×**

Prepare as in UNIT 10.20, but leave out gelatin. Autoclave and then add gelatin to 1 mg/ml. Store at −20°C.

**Oligonucleotide primers for amplification**

Prepare one pair for each target sequence at 50 pmol/µl in sterile, distilled water. Store at −20°C.

**Oligonucleotide primers for hybridization**

Prepare one primer for each target sequence and end label as in UNIT 10.10. Dissolve at 50 pmol/µl in sterile distilled water. Store at −20°C.

**Proteinase digestion buffer**

20 mM Tris-Cl, pH 7.4 (prepared from autoclaved 1 M stock; APPENDIX 2)
20 mM EDTA, pH 8 (prepared from autoclaved 0.5 M stock; APPENDIX 2)
0.5% sodium dodecyl sulfate (SDS)
Store at room temperature

**Reaction mix cocktail (per amplification reaction)**

10 µl 10× amplification buffer (see recipe)
10 µl 2 mM 4dNTP mix (UNIT 10.9)
1 µl each oligonucleotide primer for amplification (4 µl total; see recipe)

COMMENTARY

**Background Information**

PCR technology provides the most sensitive methods for detecting nucleic acids. These methods have proven exceedingly useful in the detection of infectious agents in experimental and clinical settings (Kwok et al., 1987) and in analyses of unusual and precious small samples of tissue, such as those from extinct animals (Paabo, 1989). They also have considerable potential for forensic applications (von Beroldingen et al., 1989). Most initial efforts to utilize the sensitivity of PCR were geared to issues of detection. For example, Saiki et al. (1988) showed that they could detect single target sequences (a β-globin gene) in 10⁵ to 10⁶ cells. Similarly, procedures for amplification of DNA from single cells exist (Li et al., 1988). However, these procedures were not designed to quantitate amounts of target sequence in different samples.

The basic protocol presented here to quantitate rare DNAs was developed to measure the amount of herpes simplex virus (HSV) DNA in the ganglia of mice infected with various HSV mutants. Initial efforts used slot-blot hybridization, but failed to detect viral sequences reliably below 0.01 to 0.1 copy of HSV DNA per mouse cell equivalent (Leib et al., 1989). For this reason, a number of parameters in the basic PCR protocol (UNIT 10.20) were varied to keep the replication machinery in excess to the number of templates (see Critical Parameters and Troubleshooting). Using this approach, it has been possible to quantitate HSV DNA over a 10⁴-fold range, from a few molecules per 100 ng ganglion DNA from mice infected with mutants with serious growth impairments to
tens of thousands of molecules in ganglion DNA from mice infected with wild-type virus (Katz et al., 1990). This approach should be pertinent to numerous other applications where small amounts of nucleic acid need to be detected and quantitated, including diagnostics and forensics. In fact, similar assays have been developed to quantitate nucleic acids encoded by human retroviruses (Arrigo et al., 1989; Pang et al., 1990) and to measure cytokine mRNA and DNA (Gilliland et al., 1990).

Critical Parameters and Troubleshooting
All parameters that are critical in the basic PCR protocol (UNIT 10.20) are also critical here. Several additional points particular to this protocol bear emphasis.

Optimization
The PCR must be optimized to the particular set of templates and primers and to the lot of Taq DNA polymerase used, following the guidelines in UNIT 10.20. Begin optimization with a reconstructed mixture of DNA, such as viral DNA mixed with host DNA at fairly high copy number, where both products can be visualized by ethidium bromide staining (e.g., 1 copy per cell equivalent).

DNA
The amount of added DNA is critical for achieving a quantitative assay. Too much DNA will saturate the replication machinery, not only in terms of specific products generated, but also nonspecific products. In the protocol presented above, 100 ng of DNA are used per 100-μl reaction. Test a dilution series of the sequence of interest mixed with the endogenous sequences (e.g., various copies of viral DNA per host cell equivalent) at various amounts of total sample DNA to see how much sample DNA will allow a monotonically increasing signal with increasing amounts of the sequence of interest. In addition, impurities in DNA preparations can interfere with PCR; additional organic extractions and/or ethanol precipitations in the presence of ammonium acetate may help.

Internal control
A set of primers to yield an internal control sequence is critical for two major reasons. First, if no signal from the sequence of interest is obtained in a given sample, the internal control will verify whether this is a true or a false negative. It is not uncommon for amplifications to fail, especially since some experimental samples contain contaminants that interfere with PCR. Second, the internal control allows for quantitation, since it normalizes for several factors including variation in the amount of sample DNA, efficiency of amplification, and the amount loaded on the gel. The internal control PCR product should be different enough in size to be resolved easily from the PCR product of interest, but close enough in size so there is no concern about differences in transfer efficiency due to size differences. It should also be clearly distinguished in size from an artifactual “primer-dimer” product (see UNIT 10.20).

In the protocol described here, the internal control product is derived from endogenous cellular sequences. For some applications, this would not be appropriate or practical. An alternative is to spike the samples with a known amount of a foreign sequence and to include primers specific for this DNA in the amplification reaction. This would control for the efficiency of the reaction and certain other factors, but not for variation in the amount of sample DNA.

DNA polymerase
The amount of Taq DNA polymerase added per reaction is critical in achieving a quantitative assay. The protocol outlined above uses more polymerase than the basic protocol in UNIT 10.20 to insure that the replication machinery is in excess of the number of templates. However, because Taq DNA polymerase is not the cheapest reagent, and because too much of this enzyme is actually inhibitory, the amounts of polymerase should be varied to determine an amount that permits a quantitative assay without bankrupting the laboratory.

Contamination
It is critical, in any procedure that can detect only a few molecules, to be absolutely scrupulous in avoiding contamination from exogenous nucleic acids. This protocol explicitly states several precautions one can take to avoid contamination. They are summarized below in order of importance.

Never prepare DNA or PCR reagents or assemble PCRs in the same place that is used to handle large amounts of plasmid DNAs or PCR products. PCR products are the worst possible contaminants because they give rise to more of themselves faster than normal DNA. If possible, perform the two different procedures in different rooms, avoiding even the presence of PCR products in the room where DNA is
prepared or PCRs are assembled. In addition, try preparing DNA and PCR reagents and assembling PCRs in a laminar flow hood equipped with a UV light to inactivate contaminating DNA.

If possible, use only sterile disposable plasticware for preparing and storing reagents. If glassware must be used, try to ensure that it has not been contaminated with plasmid or genomic DNAs.

Most pipetting devices can be readily contaminated by the aerosols that they produce. This protocol uses sterile disposable microcapillary pipets with a separate bulb for each reagent and sample type. This degree of caution may be extreme. A more expensive, but more convenient, alternative may be to use positive, displacement pipetting devices with disposable tip and plunger, but even then, be sure not to use the same device for handling PCR products. Recently, pipet tips with cotton plugs have been marketed to minimize aerosol contamination.

Reagents should be sterile whenever possible and stored as small aliquots.

Wear disposable gloves and change them frequently during preparation of DNA and assembly of PCR.

Be careful of aerosols from opening tubes. As outlined in the protocol above, keep all tubes closed except those receiving sample DNA. If possible, prepare only equivalent DNA samples at the same time. The use of screw-cap tubes can be helpful. It may also be useful to dedicate a microcentrifuge solely to PCR assembly.

As stated in the protocol, it is critical to include a sufficient number of negative controls (≥3) including a zero-copy reconstruction, which can help assess reagent contamination, as well as controls for DNA preparation, such as samples from mock-infected animals.

If contamination by plasmid DNAs or PCR products is suspected, it can be confirmed or ruled out using appropriately chosen sets of primers.

Aside from PCR products and plasmid DNAs, be suspicious of other sources of contamination, including various pieces of laboratory apparatus that come into contact with large amounts of target sequence. This extends to tools used to extract tissue specimens. In addition, contamination from the investigator should be avoided. Some have found that wearing a mask and hair covering is helpful. Paranoia can be a positive character trait in this situation.

Several investigators have used nucleases or UV light to inactivate contaminants in solutions. These methods can be useful but have their limitations, especially if the contaminating DNA is short and thus represents a small target.

The quantitative protocol described here can be helpful in assessing contamination because unlike nonquantitative assays, it can give an estimate of the level of contamination. Even if negative controls yield positive signals, if they are much lower than those in experimental samples this will provide at least tentative assurance that the experimental signals are real.

Transfer and cross-linking

It is very important that PCR products be transferred and retained completely to ensure a quantitative assay. If the products are electrophoresed on acrylamide, the electroblotting and UV-cross-linking protocols in UNIT 10.6 provide methods that have proven successful in this regard.

An alternative approach to transfer and hybridization is to use PCR primers prelabeled to high-specific activity with polynucleotide kinase. The products can then be quantitated by direct autoradiography of the gel (Arrigo et al., 1989; Pang et al., 1990). Potential disadvantages of this approach are that it adds steps where contamination can be introduced and detects more nonspecific products.

Anticipated Results

Results are obtained at different stages of the protocol. The DNA preparation should yield relatively intact and pure DNA, but due to the precautions described, yields may not be as high as ordinarily obtained.

After the PCR products are electrophoresed on a gel, ethidium bromide staining should reveal an easily visible band corresponding to the internal control product in every product lane. A visible band corresponding to the experimental product of interest should appear in the lane containing the 1-copy-per-cell-equivalent reconstruction and in any lanes that contain similar amounts of the sequence of interest. Because the sequences of interest are relatively nonabundant, a variety of nonspecific products may be seen as well.

After hybridization to the sequence of interest and autoradiography, the expected results are labeled bands of the appropriate size at monotonically decreasing intensities in the reconstructed dilution series, no bands in the negative controls at that size, and bands with varying intensities in the experimental samples. Depending on the probe and the stringency of the hybridization and wash conditions, nonspec-
cific sticking of the probe to the abundant internal control product may be seen. It is also common to see minor specific PCR products of slightly greater or lower mobility than the major specific product, especially at high copy number.

**Time Considerations**

The time to prepare DNA is mainly occupied by the proteinase K digestion, which usually runs overnight. Therefore, it is usually possible to start the procedure one afternoon and complete the manipulations by noon the next day. It may take somewhat longer for the DNA to dissolve completely.

Assembling the amplification reactions requires only a couple of hours or less, depending on the number of samples. Thirty cycles of denaturation, annealing, and extension require ~4 hr in a good automated cycler; slightly less if done by hand with three water baths. With an automated cycler, the gel can be poured during the cycling.

Running the gel only requires a few hours or an overnight run. Staining, destaining, and photographing requires 1 to 2 hr, electrophoresis ≥3 hr, and prehybridization and hybridization a few hours to overnight. When everything is optimal, the PCR signal from one or a few molecules can be visualized in an overnight exposure. Thus, from the point of acquiring a sample to autoradiographic signal is usually 4 normal working days.

**Literature Cited**


**Key Reference**

Katz et al., 1990. See above.

Uses the protocol outlined here and presents examples of data generated.

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