Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation

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ABSTRACT

In vitro enzymatic amplification of nucleic acids by PCR or other techniques is a very sensitive method to detect rare DNA segments. We present here a protocol that allows the rapid, sensitive and precise quantification of DNA molecules using PCR amplification run to saturation. The DNA (or cDNA) to be assayed is co-amplified with known amounts of an internal standard DNA. We show that the latter must be almost identical to the assayed DNA, otherwise quantification at the plateau is unreliable. The read-out of the amplification involves one or two additional oligonucleotides. Using fluorescent oligonucleotides as primers in run-off reactions together with an automated DNA sequencer, we could measure the level of expression of several genes, like the murine MHC class I H-2Kd or a specific T cell receptor /S chain transcript in the course of an immunization. mRNA levels were normalized by measuring in a similar manner the number of transcripts encoding the housekeeping gene HPRT. Finally, our procedure might allow the rapid analysis of a large number of samples at the same time, as illustrated by the simultaneous analysis of the mRNAs encoding the CD4 and CD8 murine T cell markers.

INTRODUCTION

DNA segments of (at least partially) known sequences can be copied in vitro with specific oligonucleotide primers and certain DNA polymerases following procedures which yield an exponential increase of the number of copies. The first, and most commonly used technique is the Polymerase Chain Reaction (PCR) (1, 2), but other techniques have been described (3-6). They offer a very sensitive means of detecting a specific sequence in a complex mixture of nucleic acids, thereby allowing the detection of rare DNA sequences (e.g. a virus infected cell among many uninfected ones) or rare RNA transcripts.

It has, however, proven difficult to make these amplification reactions quantitative, that is, to deduce accurately from the amplified products the number of DNA sequences present in the sample prior to amplification. This quantification could be an important improvement, for example in HTV viremia or in the evaluation of the number of lymphocytes infiltrating a tumor and more generally, in a large number of diagnosis tests and medical or environmental analyses. Various approaches have been developed in the last few years (7–17). Reasonably good results have been obtained when a DNA molecule of known concentration is co-amplified with the same primers in the same tube, and when the reaction is stopped during its exponential phase, well before it reaches the plateau (7–8,18). Such procedures are, however, somewhat unprecise and rather inconvenient in practice. Attempts have, therefore, been made to develop quantitative procedures involving reactions driven to the plateau (15,17). Among these quantitative methods, we can name the competitive amplification (illustrated in the PCR aided transcript titration assay (PATTY) (7) and in the procedure described by Gilliland et al. (9)) and PCR-Single Nucleotide Primer Extension assay (PCR-SNuPe) (16,19). Here, we explicitly show that, in order to perform quantification under saturating PCR conditions, the internal standards must be very similar to the DNA (or cDNA) to be assayed. Using this principle, we then describe experimental procedures which allow a relatively easy and accurate quantification under plateau conditions.

MATERIALS AND METHODS

1) Mice, cell lines and antibodies
BALB/c mice were locally bred. The Ac38 cell line originated from the fusion of CBA cells with X63Ag8 (H-2d) (20). 42F8 was derived from LPS-stimulated murine spleen cells fused with Sp2/0 (H-2d) and was a gift from A. Grandien (Institut Pasteur). X63Ag8.IL2 derived from X63Ag8.653 (H-2d) transfected with IL2 cDNA (21) and was a gift from F. Melchers (Basel Institute for Immunology). The H97-76-7 mouse monoclonal antibody recognizes the MHC class I Kd molecule (22).

2) Flow Cytometry
5.10^7 cells were pelleted, washed, resuspended in 100 μl of PBS supplemented with 5% fetal calf serum, and incubated 1 hour on ice with phycoerythrine-conjugated H97-76-7. The cells were then washed again and resuspended in 100 μl of the same buffer. Red fluorescence was measured with a FACScan flow cytometer (Beckton Dickinson) with the LYSIS II software.

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3) Plasmids

PH-2δ-33 was isolated by Lalanne et al. (23), PH2 was obtained by cloning the KpnI-Dral fragment of PH-2δ-33 into the KpnI-EcoRI site of Bluescript pBS SK+ plasmid. PH2* was derived from PH2 by HindIII-digestion, filling of the resulting 3' protruding termini with T4 DNA polymerase and ligation. PH2-diff resulted from the cloning of a non-specific PCR product using H2.3' and H2-diff primers into the Smal site of pBS SK+ (Figure 1).

PHIV was a gift from Dr Wain-Hobson and contains the V4A.15 mutant sequence of HIV-1 Tat gene (24). PHIV* was obtained from PHIV by SacI-digestion, followed by T4 DNA polymerase nibbling of the resulting 3’ protruding termini and ligation.

PHV/33* contains the sequence of the variable domain of the T cell receptor β chain of clone 5CC7 in which the 4 base pair long sequence GATA was inserted after the codon encoding Ser 81 (25).

pCD4 and pCD8 were obtained by cloning, in the vector M13mpl8, the PCR products generated with CD4.5' and CD4.3' or CD8.5' and CD8.3' primers respectively after appropriate cleavage by EcoRI and HindIII (for pCD4) or EcoRI and BamHI (for pCD8). The recombinants were then cleaved by PstI and SstI respectively, submitted to Mung Bean nuclease and T4 DNA polymerase digestion to generate blunt extremities then religated upon themselves, diius generating vectors containing the same polymerase digestion to generate blunt extremities then religated vectors used as carriers of HPRTdel and HPRT.3' into M13mpl8 previously dephosphorylated.

pCD8* was obtained by cloning the phosphorylated PCR product of HPRTdel and HPRT.3' into M13mpl8 previously dephosphorylated. All recombinants were sequenced (26).

4) Oligonucleotides

The following oligonucleotides were used:

H2.5': 5'-CTGACCTGCGAGTGTAGG-3'
H2.3': 5'-TGACTATTGCGGAGTTTGCAT-3'
H2.RO: 5'-XGAAATCCTAGCTTGAACTAAAGC-3'
H2.3': 5'-ATGGATCCATATAGACAACGAAGG-3'
H2.5': 5'-CTGACCTGCGAGTGTAGG-3'
H2.RO: 5'-XGAAATCCTAGCTTGAACTAAAGC-3'
HPRT.RO: 5'-XTTCTTTTACCGTTAGTTG-3'
HPRT.3': 5'-TGACTATTGCGGAGTTTGCAT-3'
HIV/33.RO: 5'-XACCTTGCAGCCTAGAAATTCAGTCC'-3'
HIV.5': 5'-GCAAAGATGAGGTGTATCCCTG-3'
HIV.3': 5'-GTAATGATCAGTCAACGGGGGAC-3'
V3.5': 5'-GCAAAGATGAGGTGTATCCCTG-3'
V3.3': 5'-GTAATGATCAGTCAACGGGGGAC-3'
HTV.3': 5'-CTACTACTAATGCTACTATTGC-3'
HTV.RO: 5'-XAAAGGCTTAGGCATCTCCTAT-3'
H2.5': 5'-CTGACCTGCGAGTGTAGG-3'
H2.RO: 5'-XGAAATCCTAGCTTGAACTAAAGC-3'
H2.3': 5'-ATGGATCCATATAGACAACGAAGG-3'
H2.RO: 5'-XGAAATCCTAGCTTGAACTAAAGC-3'

where X stands for the fluorescent dye (Fam) which was conjugated as recommended by the supplier (Applied Biosystems).

5) RNA and cDNA preparation

RNA from organs or from a known number of cells were purified in cesium chloride gradients (27) and reverse-transcribed, using the Boehringer cDNA synthesis kit. Occasionally, the reverse transcription efficiency was estimated by measuring α32P-dCTP incorporation.

6) PCR amplification

Amplification reactions were performed in a 50 μl mixture containing 20 u/ml of Taq polymerase (Promega), 200 μM dNTP, 0.5 μM of each of the two primers, 1.5 mM Mg++, in Promega buffer. The reaction mixtures were overlaid with 50 μl of mineral oil. Amplifications were started with a denaturation step of 1 min. at 94°C, followed by cycles (usually 40) consisting each of 1 min. at 94°C, 1 min. at 60°C, 4 min. at 72°C (to minimize intra-PCR recombination (28)), and ended up with a step of 10 min. at 72°C.

7) Run-off reaction

Two μl of the amplified solution were mixed in a final reaction volume of 10 μl containing 0.1 μM of the dye-labeled oligonucleotide, 20 u/ml of Taq polymerase, 200 μM of dNTP, 3 mM Mg++, in Promega buffer. Primer extension involved a denaturation step of 2 min. at 94°C, followed by 1 min. at 60°C and an incubation of 15 min. at 72°C.

8) Electrophoresis and analysis

The run-off reaction products were mixed with an equal volume of a 20 mM EDTA formamid solution, heat-denatured at 80°C for 10 min. and 2 μl of the resulting mixture were loaded on a 4% acrylamid, 8 M urea gel and electrophoresed for 4 hrs using an Applied Biosystem 373A DNA Sequencer. A software was specially devised to measure, for each detected DNA peak, both its length (with a precision below 0.2 nucleotide) and its area (with a precision below 5%).

When the number of single-stranded cDNA molecules was measured using a double-stranded DNA standard, the experimental values were multiplied by two.

RESULTS

1) Experimental design

a) As in other procedures (7—9), the basis is the co-amplification of the sample of interest with an internal standard DNA using the same two primers, but, in addition, we require that the sequence of the standard DNA diverges as little as possible (typically one or a few base pairs out of a few hundred) from that of the DNA to be assayed (7,15).

b) The read-out of the amplification reaction involves one or two additional oligonucleotides which allow to discriminate between the two DNA species stemming from the amplification of the sample and standard DNA respectively.

In practise, we generated the internal standards by creating a small insertion or deletion (typically 4 nucleotides out of a few hundred) in the wild type DNA sequence of the standard DNA. One convenient way was to digest the wild type DNA by a restriction enzyme creating protruding extremities, which were then either trimmed or filled by a polymerase, and subsequently religated (plasmids used as standards are labelled below with an asterix). The number of standard DNA copies was deduced from the optical density of the concentrated solution of standard DNA. After co-amplification...
H.2.5

H.2.R0

H.2.3

pH2

pH2*

pH2-diff

H2.5'

H2.RO

H2.3'

Irrelevant sequence

Figure 1. Construction of two standard plasmids for H-2K mRNA quantification. A. pH2* was derived from pH2 by insertion of 4 base pairs at the HindIII site. pH2-diff was constructed by cloning an unspecific PCR product generated with H2-diff and H2-3' primers. Due to the sequence of H2-diff, pH2-diff can still be amplified by H2.5' and H2.3' and detected using H2.R0 in a run-off experiment. B. After co-amplification of different amounts of pH2 and pH2*, a run-off reaction is initiated by the fluorescent primer H2.R0, thus generating 159 and 163 nucleotide long fragments from pH2 and pH2* PCR products respectively. After electrophoresis, the fluorescent profiles are recorded and peak areas computed.

of the standard and the DNA to be assayed, we detected the PCR products by performing a run-off step initiated with one additional labelled primer. The latter hybridized equally well to both amplified species and was nested between the primers used in the amplification in such a way that the two elongation products differed by 4 nucleotides and were therefore readily separated by electrophoresis. Here, the products were loaded on a sequencing gel and analyzed in an automated DNA sequencer (ABI). As illustrated in Figure 1B, fluorescence was recorded, and the areas of the peaks were measured. The ratio of the intensities of the two bands provided the quantification (17).

In all the experiments described below (except in 2) we performed 40 cycles of amplification, and we verified that the amplifications were effectively run to saturation.

2) Co-amplification with quasi-identical and non identical standards

We first constructed several plasmids derived from pH2, which carries a fragment of cDNA encoding the murine Kd class I histocompatibility molecule. As described in Materials and Methods and in Figure 1A, these three plasmids contained a sequence that could be amplified by the pair of oligonucleotides H2.5' and H2.3'. A third primer, H2.R0 could detect the different fragments. These PCR products contained the murine Kd class I sequence for pH2, the same sequence containing a small insertion of four bases for pH2*, and an irrelevant sequence of the same length for pH2-diff.

We asked whether the two plasmids pH2* and pH2-diff could act as internal standards in co-amplification experiments. In a first experiment, we co-amplified in two series of tubes 10^4 copies of either pH2* or pH2-diff with 10^3 to 10^5 copies of pH2. Forty cycles were performed, driving the reaction to the plateau. Results in Figure 2A show that pH2* could serve as an internal standard (9 000 copies of pH2 were detected instead of the expected 10 000). In addition, the results were highly

Figure 2. Quantification of pH2 using pH2* and pH2-diff as standard DNA. A. 10^4 copies of either pH2* or pH2-diff were co-amplified using H2.5' and H2.3' together with 10^3 to 10^5 copies of pH2. Forty cycles were performed. A run-off reaction was carried out on the amplified material using the fluorescent H2.R0 oligonucleotide. Fluorescence was recorded as indicated in Figure 1. The ratios were calculated and plotted against the number of pH2 DNA copies mixed with pH2* or pH2-diff. All experiments were done in duplicate. For each point, standard deviation is indicated. The number of copies of pH2* was determined as the abscissa of the point of the curve, whose ordinate is 1 (17). B. Around 10^4 copies of pH2 were co-amplified using H2.5' and H2.3' together with 3.10^3 or 7.10^4 copies of either pH2* or pH2-diff. Twenty-five, 30 or 40 cycles were performed. Data are represented and calculated like above.
reproducible, as can be seen from the standard deviations shown in Figure 2A.

On the contrary, the quantification was impossible when pH2-diff was used as a standard molecule. The ratio between the two amplified species did not reflect their initial ratio after a saturating number of cycles (Figure 2A), as if or near the plateau, the two sequences were amplified as two independent species.

In a second set of experiments, we analyzed the influence on the experimental values of the number of cycles performed. The quantification of a constant volume of the pH2 sample containing around 10⁴ copies was carried out using two different amounts of either pH2* or pH2-diff, and performing 25, 30 or 40 cycles of amplification. As expected, results in Figure 2B show that, when pH2* was used as the internal standard, the quantification was clearly independant from the number of amplification cycles performed. On the other hand, in no case could pH2-diff be used as an internal standard, even when as few as 25 cycles were performed (in these amplifications, plateau was found to be reached before 25 cycles).

Finally, the linearity was maintained over at least 4 orders of magnitude since similar results were obtained with samples containing as few as 10² or as many as 10⁶ molecules of pH2, provided that the scale of pH2* was varied accordingly. Usually 3, or even 2, concentrations of pH2* were enough to build a reliable reference scale provided that the approximative concentration of pH2 was known within 2 orders of magnitude. Indeed, because in these and all subsequent experiments, the slope of the reference scale was invariably one, or close to one, a single concentration of pH2 was, in principle, sufficient. However, with 2 or more, the accuracy was improved and was in the order of 25% when more than 100 molecules of pH2 were assayed.

3) Sensitivity

To check the sensitivity of the procedure, we performed a reconstitution experiment similar to that reported previously (17). We mixed cloned HIV DNA with various amounts of human DNA, and quantitated the mixture with an internal standard, pHIV* (cf. Materials and Methods). Ten, 10³ or 10⁶ copies of pHIV were subjected to 40 cycles of amplification with primers HIV.5' and HIV.3', mixed with the appropriate scaled copies of pHIV*. The experimental results closely matched the known dilutions of HIV DNA into human DNA, and 10 copies were readily assayed (data not shown).

We reasoned that several cycles of run-off reactions should increase linearly the fluorescence signal as a function of the number of cycles. This was indeed the case up to at least 10 cycles, while the ratio of the areas of the two peaks remained constant (data not shown). This procedure can, therefore, be used to increase the sensitivity of the read out, if necessary.

4) Quantification of mRNA

a) Normalization. These results show that DNA can be quantitated by the approach we described here. Once mRNA is reverse transcribed into cDNA, the same method can then be used to quantify cDNA and, therefore, mRNA. Nevertheless, RNA extraction and reverse transcription often have rather variable yields. Since the inclusion of an internal RNA standard would not check any variations in RNA extraction which are often much higher than for DNA extraction, we preferred to develop assays for housekeeping gene transcripts in order to normalize the results obtained with different RNA and cDNA samples, under the assumption that these genes were equally transcribed in the various samples and that their transcripts are reverse transcribed with comparable efficiencies. Two standard DNAs were prepared, each 4 nucleotides shorter than the corresponding mRNAs, one coding for β-actin, the other for the mouse hypoxanthine phosphoribosyl-transferase (HPRT) gene. The HPRT gene, like other housekeeping genes is expressed at very low levels in most cell types (29). In order to verify that this gene could be used as an internal control for the quantity of mRNA in our quantification experiments, we quantified the number of HPRT copies in different cDNA prepared from a known number of cells originating from three distinct cell lines as well as from a mouse spleen. The results of these quantifications are shown in Table 1. We first measured the number of HPRT coding cDNA copies present among the cDNA resulting from the reverse transcription of 2 ng of total cellular RNA, and found numbers ranging from 900 to 4400 in the different cell lines and tissues tested. Taking into account the yield of RNA extraction and of reverse transcription, we calculated that the number of HPRT mRNA per cell ranged from 5 to 10 in the three cell lines tested. We subsequently assumed that the number of HPRT mRNA copies per cell was 10.

b) Validation. Using pH2*, we then decided to quantify the number of H-2Kd transcripts in a variety of cell lines (H-2d) and tissues of BALB/c mice (H-2d) (Kd is a well characterized class I major histocompatibility antigen, which is detectable with specific monoclonal antibodies on the surface of most cells of BALB/c mice). RNA was extracted from the AC38, 42F8, X63Ag8.IL2 cell lines and tissues from BALB/c mice, and reverse transcribed into cDNA. Aliquots were mixed with pH2* or pHPT* (in separate tubes) for co-amplification and run-off. It was seen that: 1) the number of H-2Kd cDNA copies present among the cDNA obtained from the reverse transcription of 2 ng of total RNA ranged from 90 (for X63Ag8.IL2) to 57000 (for BALB/c lung), 2) upon renormalization with HPRT cDNA level, the number of H-2Kd cDNA copies detected per cell (i.e. for 10 HPRT cDNA copies), varied from 0.8 (for X63Ag8.IL2) to 290 (for BALB/c lung). The numbers of copies detected in

<table>
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<th>cell lines:</th>
<th>number of HPRT cDNA copies</th>
<th>number of HPRT mRNA copies per cell</th>
<th>number of Kd cDNA copies</th>
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<td>9</td>
<td>91</td>
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Two ng of total RNA from different cell lines or organs were reverse transcribed into cDNA. The different cDNAs were amplified in separate experiments together with the appropriate series of dilutions of pH2* or pHPT*. The number of copies of HPRT mRNA per cell was calculated under the assumption that the efficiency of cDNA synthesis was equal to 1. Finally, the number of H-2Kd mRNA copies per 10 copies of HPRT mRNA was indicated.
organized in such a way that several samples could be quantified
the PCR and run-off primers are chosen in order to generate
in a single electrophoresis step. This would simply require that
gene sequences and the corresponding standard DNAs would be
5) Quantification of several DNA or mRNA sequences
Finally, it appeared possible to build a detection system in which
immunized ones (Figure 3).
restimulation at day 49) orders of magnitude over the non-
mRNA expression, we found that the number of mRNA copies
previously described (V/J3.A', J/J2.1.2 and V/J3.B: (34)) and
J/J2.1 segments with a CDR3 coding region of 27 base pairs
chain uses the V/J3 and
a T cell clone whose T cell receptor

Figure 3. Quantification of a specific mouse TcR \( \beta \) chain mRNA in the course of an immunization. Total RNA from lymph nodes of B10.A mice immunized twice (D14) or hyperimmunized (D49) with either complete Freund adjuvant (CFA) alone or pigeon cytochrome C (PCC) mixed with CFA were prepared and reverse transcribed into cDNA (34). Various dilutions of the pV/J3* standard DNA were mixed with a fixed amount of the different cDNA, corresponding to the reverse transcription of 200 ng of total RNA, and amplified to saturation (40 cycles) with V/J3.5' and V/J3.3' oligonucleotides. A run-off reaction was performed on each of the amplified material using the fluorescent V/J3.RO oligonucleotide. As in figure 2, electrophoresis was performed, peak areas were measured and the ratios were plotted against the number of pV/J3* standard DNA mixed to the different cDNA. The number of copies were then determined as in Figure 2.

BALB/c organs were consistent with the expected values: H-2K
cDNA represent about 0.05% of all cDNAs in mouse liver (23).
On this basis, and under the assumption that a liver cell contains
some 100 000 mRNA molecules, there should be about 50
H-2K\(^d\) mRNA copies per cell in the liver. Thymus and lung
express Class I molecules at a higher level and conversely, brain
is known to express H-2K at a very low level (30), which is
consistent with our own measurements (Table 1).
We then wanted to check whether the level of expression of
mRNA coding for H2-K\(^d\) in two of these cell lines would
 correlate with the level of K\(^d\) expressed at their surface.
Cytometry experiments showed that AC38 expressed 1.7 times
more K\(^d\) than X63Ag8.IL2. This value is very close to the
ratio of mRNA expression level we have found in these two cell
lines (ratio of 1.9; see Table 1).
c) Example of an application. To illustrate the usefulness of the
procedure, we decided to study the immune response against
pigeon cytochrome C (PCC) in B10.A mice. The T cell response
against PCC has been extensively characterized (31,32) and it
is known that the response is dominated by the emergence of a
T cell clone whose T cell receptor \( \beta \) chain uses the V/J3 and
J/J2.1 segments with a CDR3 coding region of 27 base pairs
(25,33). Using this information, specific oligonucleotides
previously described (V/J3.A', J/J2.1.2.3 and V/J3.B: (34)) and
a standard DNA, pV/J3*, we were able to quantify this TcR \( \beta \)
chain mRNA together with HPRT mRNA during the course of
immunization of the animals. After normalization with HPRT
mRNA expression, we found that the number of mRNA copies
was increased by 2 (after two weeks) and 3 (after in vivo
restimulation at day 49) orders of magnitude over the non-
imunized ones (Figure 3).

5) Quantification of several DNA or mRNA sequences
Finally, it appeared possible to build a detection system in which
gene sequences and the corresponding standard DNAs would be
organized in such a way that several samples could be quantified
in a single electrophoresis step. This would simply require that
the PCR and run-off primers are chosen in order to generate

DISCUSSION
We describe here a general procedure which allows to quantify
enzymatic amplification reactions — and more particularly the
most widely used, PCR — run to saturation. It is practically
important that quantification can be performed at saturation for
at least three convergent reasons: operational simplicity (stopping
reactions in their exponential phase requires complex operations,
such as making sure that the plateau was not already reached
when the amplification was stopped), sensitivity (more material
to assay), and reproducibility (the number of cycles needed to
reach the plateau is highly variable from sample to sample and
even from experiment to experiment). In other terms, a major
problem of quantitative PCR is to dissociate quantification from
the yield. Our approach to solve this problem is based upon two
principles, namely: (a) The internal standard used for co-
 amplification is as similar as possible to the sequence to assay.
This is to ensure that the standard DNA and the sample
are amplified as a unique population under all circumstances,
irrespective of the number of cycles and, to a large extent, of
the intrinsic efficiency of the oligonucleotide primers. (b) One
or two additional oligonucleotides are used to read out the
amplification reaction. This provides a double check of the
specificity of the amplification reaction (occasional contaminants
likely, that contaminant species generated in one or the other lane of an electrophoresis gel, it becomes possible, and even the procedure recently described by Porcher et al. We believe, however, that the use of additional oligonucleotides may be important for two reasons: first, the cross-check of specificity appears dispensable: if the primers used for amplification are highly specific, there will be little, unspecific background at the plateau. If the standard DNA and the sample fulfill the first principle and differ in size, they can be directly separated by electrophoresis. Furthermore, the precise sizing performed in sequencing gels bears in itself a double check of the specificity because it is unlikely — though not impossible — that the contaminant DNA species will have the same size as that of the sample and/or the standard DNA. This is indeed the basis of a procedure recently described by Porcher et al (15). We believe, however, that the use of additional oligonucleotides may be important for two reasons: first, the cross-check of specificity is more rigorous and improves the reliability of the method. Second, if several samples are mixed and analyzed in the same lane of an electrophoresis gel, it becomes possible, and even likely, that contaminant species generated in one or the other amplification reaction, will fall within the sizes of samples or standards to be measured. We have actually observed this when we directly amplified a cDNA with the primers CD4.5' and CD4.R0 or CD8.5' and CD8.R0. When the PCR products were analyzed by electrophoresis, it appeared that the amplification with the CD8 primers had generated non-specific products of 90 nucleotide long, which corresponded to the size of the specific product generated with the CD4 primers (data not shown). Thus, mixing samples for their analysis requires very low background, and the use of additional primers and run-off reactions provides a good guarantee. Finally, the use of one or two additional oligonucleotides specific for the sample and the standard DNA opens the way to simpler detection methods, not involving electrophoresis. Several such methods, like the SNUPe procedure (19), have been described. Of interest to us is the possibility to read out the reaction with two oligonucleotides overlapping the few nucleotides which differentiate the standard DNA from the DNA to be assayed, a procedure which could be adapted to a direct enzymatic read out. Such procedures may be important for future routine tests involving PCR or other amplification methods. However, one should be aware that the introduction of differential treatments of the two DNA species could in fine result in either a weaker precision, or in a greater complexity.

Using the run-off procedure, we have shown here that we could quantify DNA and mRNA reverse-transcribed into cDNA. For these mRNA measurements, we thought it useful to include an additional internal control, namely the quantification of mRNAs coding for house-keeping proteins (HPRT or β-actin), in order to normalize the RNA extraction and reverse transcription yields. To that end, other groups have chosen to use an internal standard made as RNA, which would then be co-reverse transcribed prior to co-amplification (8). We reasoned that this strategy would in no way correct variations in RNA extraction efficiency and RNA quality, which are known to vary according to both the samples and methods used. In addition, standard RNA solution would be much more difficult to store and use in routine experiments.

**Figure 4.** Simultaneous analysis of several mRNA's in a single cDNA preparation. A. Fluorescence peaks obtained upon electrophoresis of the mixture of the run-off products generated by the co-amplification of 20 ng of mouse thymus mRNA with either 100 copies of pCD4* or 500 copies of pCD8*. The lengths (in nucleotide) of the products are 96 for pCD4*, 100 for CD4 mRNA, 106 for pCD8* and 110 for CD8 mRNA and are readily identifiable. B. Quantification of the CD4 and CD8 mRNA in 20 ng of total mRNA from a mouse thymus. The amounts were calculated as above.
than DNA solution, due to its greater sensitivity to nucleases and degrading agents.

Among several examples, we measured the amount of mRNA encoding a mouse major class I transplantation antigen (K\(^{b}\)) in several cell lines and found good correlation with its surface expression assessed by FACS measurements. Also, it was possible to distinguish RNAs and their internal standards, to use primers producing run-off products with a known hierarchy of sizes. As an example, we showed that the mRNAs coding for the T cell surface markers CD4 and CD8 could be quantified in a single electrophoretic step. We chose spacings such that a couple of run-off products corresponding to mRNAs and their internal standards, occupies 10 nucleotides. Therefore, at least 25 couples could be mixed and subjected to electrophoresis in one lane of the automated sequencer. Because 4 fluorescent tags and 36 lanes can be used in an instrument like the ABI sequencer, it should be possible to simultaneously analyse 3600 couples. Assuming that the assay of one DNA or mRNA requires the analysis of 3 couples (with variable concentrations of the standard DNA), some 1200 samples could be quantified in one run. Even with the inclusion of a variety of controls, the analytical potential appears to be large. We are currently building a kit which should allow the quantification of 25 distinct mRNAs in a single sample.

In summary, we have described an approach which allows to measure, with an accuracy of about ± 25%, the amount(s) of one or several DNA sequence(s) and/or mRNAs present in a biological sample, using amplification reactions such as PCR run to saturation. Such methods are expected to prove extremely useful in a large variety of situations. We have shown here that HIV sequences could be quantitatively and with great sensitivity assayed in this way; that, for the first time, the emergence of a specific clone of murine T cells could be quantified in the course of an immune response; and that the CD4 and CD8 murine T cell markers could be readily assayed and simultaneously analysed. The latter two tests should be helpful to analyse a variety of pathological situations in man (such as the nature of T lymphocytes infiltrating a tumor or an organ subjected to autoimmune attack). Several additional tests are currently being developed.

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