HIV

A Practical Approach

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Sequence analysis of virus variability based on the poymerase chain reaction (PCR)

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1. Introduction

During most stages of HIV infection, the viral population contains a large number of closely related variants that have diverged within the indvidual over the course of HIV infection. For example, *env* sequences of human immunodeficiency virus in lymphocytes of infected individuals may show variability of up to 10% within the same sample (1, 2). Under these circumstances, sequence analysis of PCR products amplified from such samples is often unreliable due to ambiguities in the sequencing gel. While there are methods for measuring the relative frequencies of alternative nucleotides at these polymorphic sites (see section 3), this does not distinguish the linkage relationships of the variant nucleotides so it is impossible reliably to reconstruct the constituent sequences.

An even more serious problem is encountered when attempts are made to read a 'consensus' sequence in areas of the HIV genome where sequences differ from each by the presence of insertions or deletions of nucleotides (e.g. the V1, V2, V4, and V5 hypervariable regions of the *env* gene). The presence of variants that differ in length make a consensus sequence completely unreadable downstream of the position of the point of the deletion/insertion. On the other hand, the presence of length variants allow different viral populations to be rapidly compared, by amplification of a sample of the virus population across the hypervariable regions (3). Methods for analysing the length profiles of whole virus populations *in vivo* are described in section 3.

2. Amplification of viral sequences

2.1 Limiting dilution assays

There are two methods for isolating single viral sequences to enable the analysis of heterogeneous samples. In the first method, cDNA or proviral DNA is amplified by the PCR followed by cloning of the PCR product into

a plasmid (e.g. pUC) or bacteriophage (e.g. M13) vector. Sequencing can then be carried out on individual clones isolated by plating out the library in *E. coli*. Most of the problems encountered with this technique are associated with inserting blunt, non-phosphorylated DNA into a vector, and inaccuracies within the nucleotide sequences that are inevitably introduced by the amplification and cloning of viral sequences with certain DNA polymerases (see section 2).

These difficulties led us to develop an alternative method for obtaining single sequences from a heterogeneous sample, in which proviral DNA or cDNA present in a sample is diluted sufficiently *prior* to amplification to ensure that only single target sequences are amplified in each reaction. To separate the sequences, it is necessary to amplify DNA in multiple replicates at a dilution where only a small proportion of the reactions yield amplified DNA. As the distribution of very dilute DNA between tubes by macroscopic pipetting is a random stochastic process, the Poisson formula can be used to calculate the likelihood of positive tubes having originally contained one, or more than one molecules of target DNA (*Table 1*). When the frequency of PCR-positive reactions is 0.2, approximately 95% of reactions can be predicted to have originated from single target sequences, whereas only half of reactions will be derived from single sequences when the frequency of positives is 0.7.

This method of separation is critically dependent on the reliable detection

Table 1. Quantitation and separation of sequences by limiting dilution

Observed frequency positives	Calculated number DNA sequences[a]	Proportion single copies[b]
0.001	0.001	99.9%
0.01	0.010	99.5%
0.05	0.051	97.5%
0.10	0.105	94.8%
0.15	0.163	92.1%
0.20	0.223	89.3%
0.25	0.288	86.3%
0.30	0.357	83.2%
0.40	0.511	76.6%
0.50	0.693	69.3%
0.60	0.916	61.1%
0.70	1.204	51.2%
0.80	1.609	40.2%
0.90	2.302	25.6%
0.95	2.996	15.8%

[a] Actual frequency, f (in target molecules/replicate tube) calculated according to the formula $f = -\ln(f^0)$, where (f^0) is the observed frequency of negative reactions.
[b] Proportion of positive replicates, f^1 derived from a single copy of target DNA.

of single molecules of the appropriate HIV sequence. While it is possible to detect positive reactions by hybridization of the PCR with a probe of high specific activity (4), the maximum sensitivity of such methods is generally around 3–100 copies of template DNA (5–7). We use the more sensitive nested PCR approach (*Protocol 1*). A nested PCR can produce sufficient amounts of DNA from a single template molecule for sequence analysis. For example, a 300 bp target sequence has a molecular weight of 10000 g/mol and a mass of 0.016 ag (1 ag = 10^{-18} g); amplification by a factor of 10^7 with outer primers, and 10^6 with inner primers would produce 160 ng of PCR product. In contrast, a single amplification of one copy of target DNA by as much as 10^8-fold would produce barely enough product to be detectable by the most sensitive hybridization techniques (1.6 pg DNA).

Protocol 1. Amplification of DNA by nested PCR

Equipment and reagents

- PCR buffer: 200 mM Tris–HCl pH 8.8, 500 mM KCl, 15 mM $MgCl_2$, 0.5% Triton X-100
- Nucleotide triphosphates (100 × stock): 3 mM each of dATP, dCTP, dGTP, dTTP
- 15–60 μM sense primer (for a 20-mer, 100–400 μg/ml)
- 15–60 μM antisense primer
- Mineral oil (BDH, Cat. No. 29436)
- *Taq* polymerase (Cetus, Promega, Northumbria, Boehringer)
- Programmable thermal cycler
- Protective goggles, or (preferably) full face mask
- 1.5% agarose[a] gel in 1 × TBE, pre-stained with 0.66 μg/ml ethidium bromide (*note: ethidium bromide is highly mutagenic, always wear gloves when handling gels or solutions*)
- 1 × TBE electrophoresis buffer, containing 0.66 μg/ml ethidium bromide
- Size markers spanning expected size of second PCR product (e.g. plasmid DNA, such as pBR322 digested with *Hae*III, Boehringer Cat. No. 821705)
- Ultraviolet light box

Method

1. For each sample, pre-mix:
   - 5 μl PCR buffer
   - 38 μl water
   - 0.5 μl nucleotide triphosphates
   - 0.25 μl sense primer (approx. 4–16 pmol)
   - 0.25 μl antisense primer
   - 1 U *Taq* polymerase (0.1–0.25 μl at most manufacturers' supplied concentrations)
   - 5 μl DNA or cDNA sample (diluted, if appropriate)

   For amplifying multiple samples, it is easier to make up a stock containing all but the sample, and adding 45 μl to each tube. Cover with a drop of mineral oil.
2. Transfer immediately to a thermal cycler set with the following temperatures and times: 94°C 35 sec; 50°C 40 sec; 68°C 150 sec.

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