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An isothermal gene amplification method

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CELLS on microscopic slides don't like cycling. It is difficult to promote primer entrance and to prevent diffusion of amplification products. The author describes a gene amplification technique applied to detection of the HIV and which can be implemented without programmable thermocyclers. The reaction products are high molecular weight strands of DNA that can be detected using simple methods, even without electrophoresis. This is a promising technique for *in situ* amplification experiments.

Our findings show that this amplification method is effective at physiological temperatures and is compatible with the conditions encountered in living cells. This technology could thus be the basis for a new class of drugs intended for the treatment by gene therapy of certain diseases, including malaria and AIDS.

In order to develop a gene amplification method that can be used at ordinary temperature, we have tested pairs of special primers intended to be the substrate for a DNA polymerase. These primers contain a specific portion of a target DNA and another part including a palindromic sequence, enabling the primer to adopt a hairpin structure. The specific parts of the target DNA surround a DNA region of several hundred base pairs long.

The expected reaction is shown in Fig. 1. The target DNA is heated briefly in the presence of primers and DNA polymerase (Fig. 1a) then the tube is placed in a water bath at about 60°C for 3 minutes. The primers hybridize with the target DNA and DNA polymerase starts elongating new strands from the primers (Figs. 1b, 1c). The next step is another very brief heating at 100°C (Figs. 1d, 1e). The newly synthesized strand dissociates and in turn is used as the target for a new priming that will be used to synthesize a new strand. After this step, the reaction is run at constant temperature (which is dependent upon the sequence of the primers).

One primer binds to the newly synthesized strand and another strand is polymerized (Fig. 1g). When the enzyme comes to the hairpin region, it displaces the strand corresponding to the palindromic region and continues polymerization. Once a certain temperature is reached, an equilibrium develops between the linear form of the DNA formed and a form in which two hairpins are formed (this equilibrium occurs over a broad range of temperatures, but is more or less shifted towards one or the other form).

Starting at about 50°C, equilibrium is reached very rapidly (Fig. 1h). The 5' end of one of the two hairpin regions is free, and can be used to prime DNA polymerase. The enzyme synthesizes a new strand and displaces the complementary strand until it detaches (Fig. 1i). A new primer binds to this strand and the cycle starts again. During that time, synthesis terminates on the first strand and the equilibrium between the linear form and the two hairpin form is established (Fig. 1j). One of the two hairpins is used to

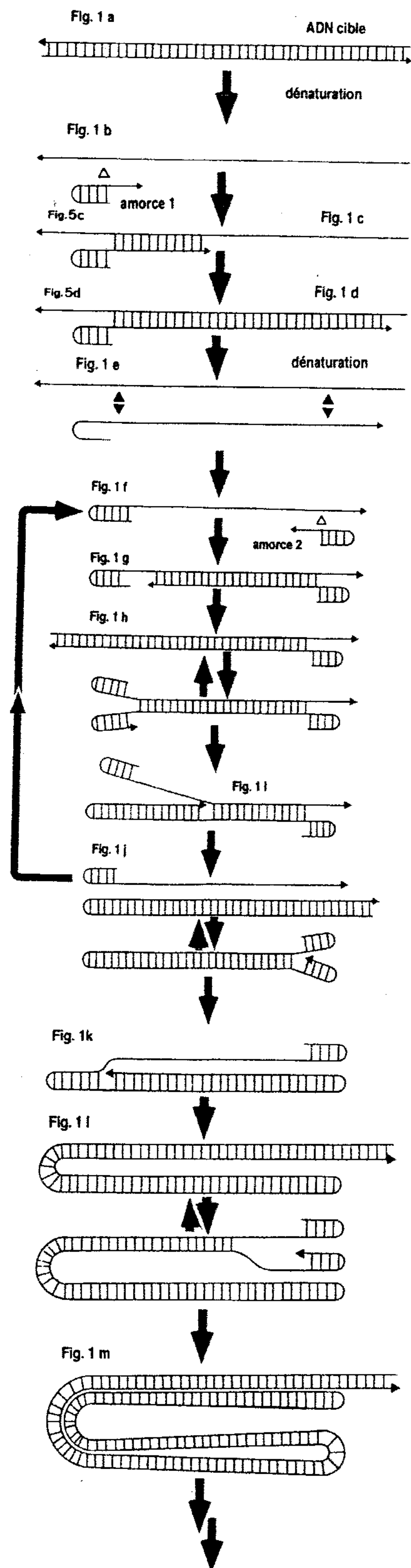

Figure 1. Détail de la réaction.

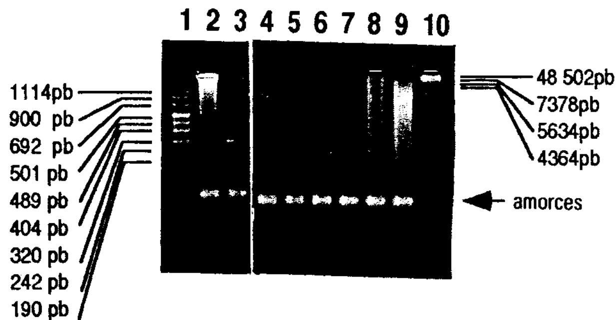

FIG. 2 Amplification of DNA with atto1 and atto 2 primers. Lane 1: mark VIII DNA marker (Boehringer). Lane 2: HIV positive serum. Lane 3,4,5,6,7: serum with negative reaction. Lanes 8 and 9: HIV positive serum. Lane 10: raoul DNA marker (Appligene)

MATERIAL AND METHODS. Primers: Labi1 (Eurogentec).
----CTGGTCATACGAACTACATTTCAGA 5'
GTAACCAGTATGCTTGATGTAAAGTCTATAATCCACC
TATCCCAGTAGGAGAAAT 3'

The Labi1 primer is composed of one part containing 26 base pairs specific for a GAG gene sequence of the HIV (1,2,3,4) and of another palindromic part (randomly chosen), of 50 base pairs, able to form a hairpin. The hairpin portion is located at the 5' end of the primer.

Labi2 (Eurogentec).
------CGTCAGAATTCTTCACTAATTAGC 5'
GCTAGCAGTCTTAAGAAGTGATTAATCGTTTGGTCCT
TGTCTTATGTCCAGAATGC 3'

The second primer (Labi2) is similar, but the HIV-specific portion of 28 base pairs is complementary to a sequence located 60 base pairs further on and oriented in the opposite direction. The melting temperature of the palindromic sequences (calculated using the Wallace rule)(5) is 66°C and that of the specific sequences is 78°C.

The products used to carry out gene amplification were furnished by Perkin-Elmer, except for gelatin: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (Sigma Cat. No. G2500), 200 mM each of dNTP. A non-thermostable DNA polymerase can probably be used, but initial experiments were conducted with Amplitaq DNA polymerase at 2.5 units/100 microliters.(6, 7) Labi1 and Labi2 primers were used at 0.5 μM each, the HIV matrix consisted of 10^4 copies per tube (Positive samples Perkin-Elmer (8)) and paraffin oil (Sigma) was used at 60 μl per tube.

In order to verify the specificity of the reaction, control hybridizations (9, 10, 11) were carried out with Bert1 oligonucleotide (Eurogentec):
5' ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC 3'

This oligonucleotide was used to hybridize with nitrocellulose membranes obtained with the automatic MB 24 Multiblotter (Labimap). This apparatus automatically carries out electrophoreses followed by membrane transfer. All operations are automated for perfect reproducibility : pipetting samples, application on the gel, migration and membrane transfer. (12)

Probes were labeled with alpha P32 dCTP (Amersham) and an Amersham labeling kit.

atto 1(Genosys):
ATA T ATATATATATATATATATATATATATATATATA 5'
TATATATATATATATATATATATATATATATATAATCCACCTATCCCAGT
AGGAGAAAT 3'

atto 2 (Genosys):
A T ATATATATATATATATATATATATATATATATA 5'
TATATATATATATATATATATATATATATATATATTTTGGTCCTTGTCTTAT
GTCCAGAATGC 3'

Other primers were also designed : Atto 13 (Gibco BRL):
5'AGACTTACTTAGCGTACTAAGTAAGTCTATAATGCACCTATCCCAGTAG
GAGAAAT3'and atto 23 (Gibco BRL):
5'CGATTATAGTTCGCTAGAACTATAATCGTTTGGTCCTTGTCTTATGTCC
AGAATGC3'and Pan-Ac16:
5'GAGTTGTTAACAGTCGTCGAACGACTGTTAACAACTCGTTTGCAGCTC
TGTGCATA 3'
Pan-Ac HPV:
5'TTAGATGAACGATAGTCCTCGAAGGACTATCGTTCATCTAAAAGGGCG
TAACCGAAATCGGT 3'
and Pan-Ac18:
5'ATGAGTTGTTAACAGTCGTCGAACGACTGTTAACAACTCATGTGTTTCA
GTTCCGTGCACA 3'

The latter three primers are intended to detect the HPV 16 and HPV 18 viruses. (13)

prime the enzyme again and a new strand is synthesized, that displaces the older one (Fig. 1k). At each cycle, the size of the newly synthesized DNA is two-fold.

Given the rate of DNA synthesis at 55°C (several hundred base pairs per second), the size of reaction products increases rapidly. As a result of the cycle described above, the number of newly synthesized DNA molecules also increases very rapidly.

Determination of the optimal temperature for the gene amplification reaction

The optimal reaction temperature for a given pair of primers is *a priori* an unknown parameter. The temperature must be sufficiently high to enable the equilibrium shown in Fig.1h, Fig.1j and Fig.1l to occur, i.e. the temperature must enable the double strand to open, but also the reformation of a sufficiently stable hairpin structure, and above all the hybridization of primers on the target DNA. It can now be seen that the melting temperature of the specific portion of the primers must be higher than the melting temperature of the palindromic portion.

To our knowledge, no computer program sufficiently efficient to predict the optimal reaction temperature for a given pair of primers is currently available. We thus built an apparatus to determine this temperature. Once this parameter is determined, a simple water bath adjusted to this temperature is sufficient to conduct all amplification experiments using this pair of primers.

The instrument is composed of a heating ramp pierced with holes in which 200 microliter tubes are placed. A temperature gradient is created along this ramp by a 60 W resistor welded to one the ends. The ramp is machined in an aluminum bar. This device is similar to the Koffler apparatus used by chemists to determine the melting point of compounds. The curve of the temperature vs. tube number was plotted and it was verified that it remained constant with time

In order to avoid contamination from the water bath liquid, we used another apparatus consisting of a heating device formed from a aluminum block pierced with 96 holes to receive the microtubes. The temperature of the block was maintained perfectly constant.

Before starting the experiments, we feared that the enzyme would not copy the strands in their entirety. After a restricted number of cycles in the gene amplification reaction, this would have stopped the reaction. Fortunately, if this effect occurred, it did not prevent the reaction from proceeding. Nevertheless, in order to counter this risk, it was planned to use primers containing a hairpin portion composed of a poly-AT sequence. (14) This portion should remain functional for a long time, even in the case of a defective copy by the enzyme. Furthermore, there may be a rolling circle type of increase, that will not be discussed in detail, but which could lead to an even further increase in yield.

RESULTS

10^4 copies of the plasmid containing the HIV genome (8) were incubated in the wells of the temperature gradient ramp between 45 and 88°C. After two hours, the contents were deposited on a 2% agarose gel containing éthidium bromide. (table I). The results are summarized in Table I.

[table I

A clearcut smear was observed in wells 7, 9 and 10.

In comparison to the Raoul molecular weight marker (Appligene), it was estimated that the size of the DNA strand products was included between 300 and 7000 base pairs, with a maximum concentration around 6000 bp. The optimal reaction temperature was thus 55°C. It was difficult to determine the quantity of DNA, since it was not concentrated in one band as was the molecular weight marker DNA. It was estimated at 0.5 micrograms.

The following experiments with this pair of primers, designed to determine the best reaction mixture composition, were run at 55°C. Agarose gel electrophoresis was followed by membrane electrotransfer in order to verify that the amplified DNA indeed contained HIV sequences (data not shown). These operations were conducted with a Labimap Multiblotter MB 24 automatic apparatus (13).

Optimal temperature was different with other pairs of primers. Using atto 1 and atto 2, this optimal temperature was 80°C. Using this pair, the mean size of reaction products was very high, to the extent that they were trapped on the top of the gel. Mean size was more than 100 kb (Fig. 2). It was surprising to observe the presence of a low molecular weight DNA, less than that of the primers, which was probably a breakdown product. The photo shows that the quantity of DNA formed was undoubtedly higher than 10 µg.

This high molecular weight DNA did not diffuse out of the gel when it was dialyzed, in contrast to the primers. We have shown that merely mixing the amplification products with a 2% agarose solution leads to a 1% final solution, that can be poured into a 96-well microplate. After incubating the microplate in a large volume of Tris 10-1, pH 8 containing ethidium bromide, positive tubes are clearly distinguished under ultraviolet light, without the necessity of carrying out an electrophoresis (data not shown). It is preferable to use a black plastic microplate with low intrinsic fluorescence. The gene amplification reaction can also occur in this microplate, and 2% agarose is added at the end of the reaction. (14)

When the PCR is used, problems of false positives due to aerosol contamination often occur and we have been faced with this problem. Because of the extreme sensitivity of the method, the precautions used in laboratories conducting PCR must be complied with.

In the case of some primers, the reaction initiated by two passes at 100°C continues to occur at room temperature (20°C), but products appear in detectable quantities only after several hours.

We made the surprising observation that after several days in a cold room at 4°C, gene amplification reaction products could be detected in a tube containing all the reagents and a positive control. (data not shown)

Although we did not use a non-thermostable DNA polymerase, this is probably possible. In the PCR technique, this enzyme is destroyed by repeated passes at 100°C for 3 minutes. In the technique we describe, the tubes are heated to 100°C only twice for few seconds. This brief treatment assures that high enzymatic activity persists, even when the DNA polymerase is non-thermostable. In this case, higher quantities must be added to the reaction tubes.

Future developments:

We believe this method to be a promising one for the improvement of *in situ* gene amplification techniques. Although we have not verified it, it is possible that the reaction may occur in living cells. The theoretical requirement is that a sufficient number of nuclease-resistant primers can penetrate the cell membrane to trigger the gene amplification reaction, filling the nucleus (or cytoplasm) with high molecular weight DNA. DNA polymerases are present in nuclei alongside proteins that open the double helix and promote recombination. It is thus possible that the reaction can initiate without the two 100°C heating steps.

It is probable that cell death will result from the application of this method, and so it may be possible to specifically eliminate cells containing a particular DNA sequence, e.g. the HIV genome, or to specifically kill parasites, e.g. *Plasmodium*, the agent of malaria.

In order to verify this hypothesis, we are planning to test primers Pan-Ac 16/Pan-Ac HPV, and Pan-Ac 18/Pan-Ac HPV, on three type of cells in culture : normal fibroblasts, HeLa cells and Caski cells. HeLa cells are cancer cells obtained from a uterine cervical cancer. These cells contain many copies of the type 18 Human Papillomavirus genome included in their own genome, and should be killed by primers Pan-Ac 18/Pan-Ac HPV. Reciprocally, (16, 17) Caski cells should be specifically killed by the primer pair Pan-Ac 16/Pan-Ac HIV. (Caski cells contain approximately 600 copies of the type 16 Human Papillomavirus genome included in their DNA.)(18, 19) We hope that normal cells will resist the treatment.

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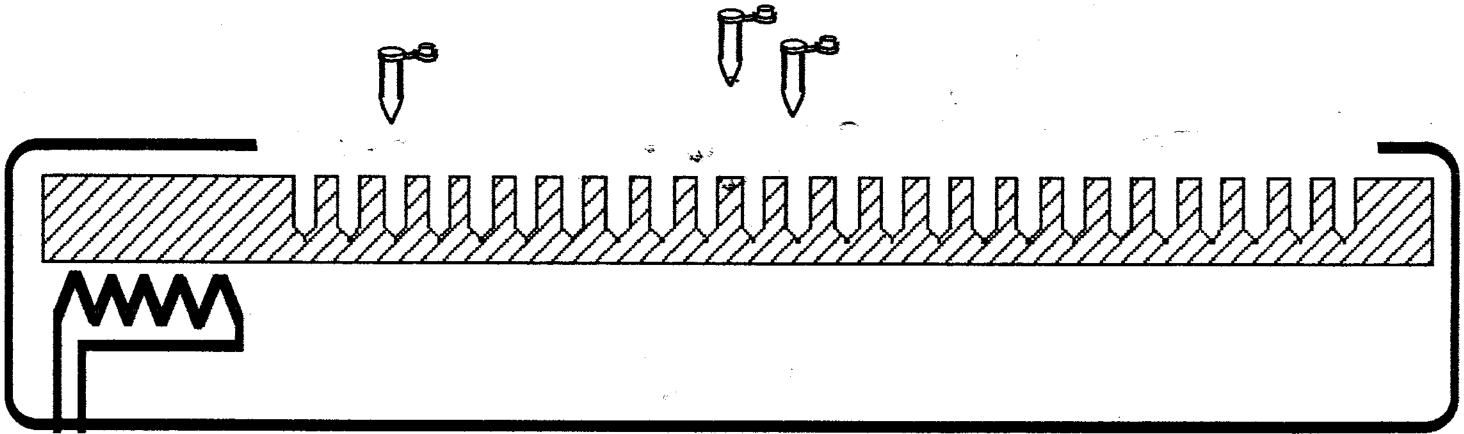

Temperature gradient heater

Tableau I. Résultats de l'incubation du plasmide contenant le génome de VIH.

Puits	Température (°C)	Résultats
1	45	–
2	46	–
3	48	–
4	49	–
5	49	–
6	50	–
7	51	+
8	52	–
9	52	+
10	53	++
11	54	–
12	56	+
13	57	–
14	61	–
15	64	–
16	65	–
17	68	–
18	70	–
19	72	–
20	76	–
21	78	–
22	84	–
23	86	–
24	88	–

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Microbiologie / *Microbiology*

Une méthode d'amplification génique isotherme

An isothermal gene amplification method

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Abstract – A gene amplification technique applied to the detection of HIV and which can be implemented without programmable thermocyclers is described. The reaction products are high molecular weight strands of DNA that can be detected using simple methods, even without electrophoresis. This is a promising technique for in situ amplification experiments. Our findings show that this amplification method is compatible with the temperature levels encountered in living cells. This technology could thus be the basis for a new class of drugs intended for treatment by gene therapy of certain diseases, including malaria and AIDS. (© Académie des sciences / Elsevier, Paris.)

genetic amplification / gene therapy / HIV / AIDS / malaria / HPV / carcinoma in situ

Résumé – Nous décrivons ici une technique d'amplification génique appliquée à la détection du virus VIH pouvant être mise en œuvre sans l'aide de thermocycleurs programmables. Les produits de réaction sont constitués de brins d'ADN de haut poids moléculaires et peuvent être mis en évidence par des méthodes simples, éventuellement sans électrophorèse. Cette technique est prometteuse pour les expériences d'amplification in situ. Selon nos observations, cette méthode d'amplification est compatible avec les conditions de température rencontrées dans les cellules vivantes. Cette méthode pourrait donc être à la base d'une nouvelle classe de médicaments destinés à la thérapie génique de certaines maladies, incluant le paludisme et le sida. (© Académie des sciences / Elsevier, Paris.)

amplification génique / thérapie génique / VIH / sida / paludisme / HPV / cancer du col

Abridged version (see p. 913)

1. Introduction

L'invention de la technique d'amplification génique a bouleversé de nombreux domaines de la recherche en biologie [1–3]. De nombreux efforts ont été engagés ces dernières années de façon à mettre au point une méthode pouvant être mise en œuvre à température constante [4–10]. Dans ce but, nous avons testé des couples d'amorces particuliers destinés à servir de substrat à une ADN polymérase. Ces amorces présentent une partie spécifique d'un ADN cible, et une partie comportant une séquence palindromique, permettant à cette amorce d'adopter une structure en « épingle à cheveux ». Les parties spécifiques encadrent une région de l'ADN cible longue de quelques centaines de paires de bases.

La réaction attendue est détaillée sur la *figure 1*. On chauffe brièvement l'ADN cible, en présence des amorces et de l'ADN polymérase (*figure 1a*) puis on place le tube dans un bain-marie à environ 60 °C pendant 3 min. Les amorces s'hybrident à l'ADN cible et l'ADN polymérase commence l'élongation d'un nouveau brin à partir des amorces. (*figure 1b* et *1c*) On chauffe très brièvement une deuxième fois et dernière fois à 100 °C (*figure 1d* et *1e*) Le brin néosynthétisé se dissocie et sert à son tour de

Note présentée par François Gros

* Correspondance et tirés à part

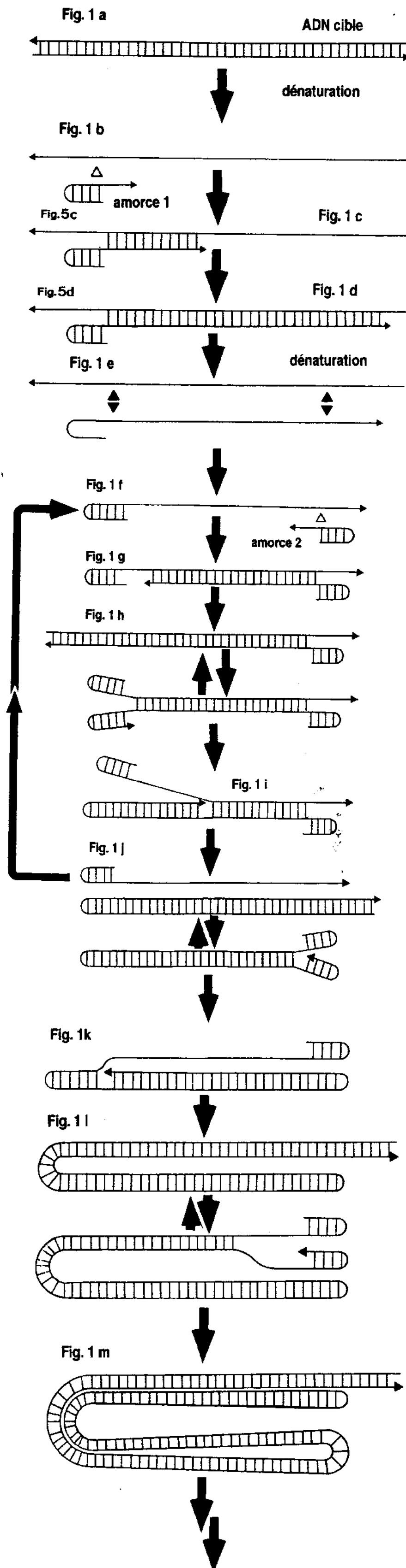


Figure 1. Détail de la réaction.

cible à une nouvelle amorce, qui va servir à synthétiser un nouveau brin.

À partir de cette étape, la réaction a lieu à température constante (cette température dépend de la séquence des amorces).

Une amorce s'est fixée sur le brin néosynthétisé et un autre brin est polymérisé (*figure 1g*). Arrivé à la région en épingle à cheveux, l'enzyme déplace le brin correspondant à la région palindromique et continue la polymérisation.

À une certaine température, nous avons un équilibre entre la forme linéaire de l'ADN formé, et une forme où deux épingles à cheveux se sont formées (cet équilibre a lieu dans une large gamme de température, mais il est plus ou moins déplacé vers l'une ou l'autre forme).

À partir de 50 °C environ, l'équilibre est atteint très rapidement (*figure 1h*). L'une des deux régions en épingle à cheveux possède une extrémité 3' libre : elle peut servir d'amorce pour l'ADN polymérase. Celle-ci synthétise un nouveau brin et déplace le brin complémentaire, jusqu'à que celui-ci se détache (*figure 1i*).

Sur ce brin, une nouvelle amorce se fixe et le cycle recommence.

Pendant ce temps là, la synthèse s'est terminée sur le premier brin et l'équilibre entre la forme linéaire et la forme comportant deux épingles à cheveux s'est établi (*figure 1j*) : l'une des deux épingles à cheveux sert de nouveau d'amorce à l'enzyme et un nouveau brin est synthétisé déplaçant l'ancien (*figure 1k*). À chaque cycle, la taille de l'ADN synthétisé double.

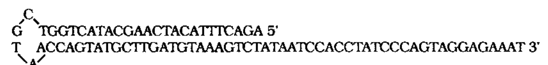
Étant donné la vitesse de synthèse de l'ADN à 55° (plusieurs centaines de bases par seconde), la taille des produits de réaction augmente rapidement. À cause du cycle décrit plus haut, le nombre des molécules d'ADN néosynthétisées augmente lui aussi très rapidement [11].

2. Matériels et méthodes

2.1 Réactifs

Les composants utilisés pour effectuer la réaction d'accroissement génique sont fournis par Perkin-Elmer : 10 mM Tris HCl pH 8,3, 50 mM KCL ; 1,5 mM $MgCl_2$; 0,01 % gélatine (Sigma cat n° G2500) 200 mM de chaque dNTP ; il est possible d'utiliser une ADN polymérase non thermostable, mais les premières expériences ont été conduites avec l'enzyme Amplitaq ADN Polymérase 2,5 Unit/100 mL ; primer Labi1 and Labi 2 : 0,5 mM de chaque. ; matrice VIH : 10^4 copies par tube ; huile de paraffine : 60 µL par tube (Sigma)

2.2. Amorces : Labi1 : (préparé par Eurogentec)



L'amorce Labi1 est constituée d'une partie de 26 nucléotides spécifique d'une spéquence du gène GAG du