DNA hybridization : comparison of liquid and solid phase formats

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SUMMARY

In nucleic acid hybridization an oligo- or polynucleotide probe is allowed to anneal to its complementary strand which possibly is present in the sample. This offers an extremely specific way to identify and quantify given genes and thus, for instance, given microbes. The annealing reaction is, however, slow since the reactants are present at very low concentrations and the diffusion rate of DNA is slow. To overcome this problem high concentrations of probe are used in order to « drive » the reaction in a pseudo-first order fashion. As a result a positive hybridization is easily masked by the large excess of unreacted probe molecules, uneless a powerful fractionation system is used which removes the free probes. A frequently used method is to immobilize the nucleic acids of the sample on a solid support which then after the reaction is easy to wash. The solid support introduces, however, a diffusion barrier which signifanctly reduces the reaction rate. Thus kinetically solution phase reactions are preferrable to solid phase ones. In this communication a test format is described in which the advantages of both solid and solution phase assays are combined. Two probes are used, one carrying a detectable lable (the detector probe) and the other an affinity moiety, e.g. biotin (the capture probe). After hybridization in solution a sandwich hybrid is formed in which the target nucleic acid is annealed to the two probes. By exposing the reaction mixture to an affinity matrix carrying the other moiety of the affinity pair, e.g. avidin, the capture probe becomes bound only when associated to the capture probe via the sandwich hybrid. Thus a quantitative measure of target is obtained in a test format which is both easy to use and relatively fast. The affinity capture method is also applicable to the quantification of PCR products.

INDEX TERMS : Hybridization - Nucleic acid probes - Laboratory diagnosis.

RÉSUMÉ

Hybridation de l'ADN : comparaison des méthodes en phase liquide et en phase solide. – Dans une réaction d'hybridation entre brins d'acide nucléique, les deux molécules s'assemblent l'une à l'autre en amorçant une réaction d'appariement de bases très rapide. La cinétique de réaction est de ce fait d'ordre 2. Les concentrations des réactifs peuvent être très faibles, de l'ordre de la femtomole, et conduisent à des réactions lentes. Lors des expériences d'hybridation, ce problème est résolu par l'addition d'un large excès de la sonde, avec dans ce cas une cinétique de pseudo ordre 1 et une vitesse de réaction augmentée. Se pose alors le problème de la séparation de l'excès de sonde de la sonde hybridée. La sonde libre, d'une concentration d'un million de fois supérieure, devra être séparée de l'hybride à l'aide d'une technique biochimique. L'acide nucléique cible est le plus souvent immobilisé sur un support solide avant hybridation, assurant ainsi une séparation efficace mais cela entraîne l'apparition de réactions non spécifiques et la diminution de la vitesse d'hybridation due à la présence de barrières de diffusion. Les auteurs ont combiné la rapidité de réaction en solution avec la puissance de séparation des techniques en phase solide. En ajoutant à la sonde de révélation marquée une deuxième sonde dite de capture, un hybride sandwich est réalisé au cours de la réaction d'hybridation. Grâce à cette sonde de capture, les hybrides peuvent être séparés de la sonde libre avec l'efficacité requise. Le choix de la méthode dépend d'un certain nombre de facteurs tels que le but de l'analyse, la concentration de l'analyte, l'efficacité-cout, les limitations de temps et le marqueur utilisé. Les possibilités d'automatisation sont importantes dès qu'il est question de tests diagnostiques de routine.

MOTS CLÉS : Hybridation - Sonde de capture - Phase solide.

The possibility to identify and quantify nucleic acids, either DNA or RNA, in various biological samples is of great value both in basic and applied research. For this aim, the hybridization reaction, in which two complementary nucleic acids strands anneal to form a double-stranded structure, is used. This reaction is very specific, since the probability of random nucleotide sequences to be complementary over significant regions is practically non-existing. The hybridization reaction obeys second order reaction kinetics. In many cases the concentration of the nucleic acid to be analyzed, i.e. the target, is extremely low. Hence the reaction rate becomes slow. To overcome this problem a large excess of probe molecules is added to « drive » the reaction, in a pseudo-first order fashion [1]. The large excess of labelled probe in the assay introduces the problem of separating free probe from hybridized probe. In this communication methods by which hybridization assays can be made fast and convenient, without losing sensitivity are discussed.

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Immobilized or soluble target ?

In most applications of nucleic acid hybridization the target molecule is immobilized onto a solid support, such as a filter. The immobilized target is then allowed to react with the labelled probe. The advantages of this approach are :

– the two strands of a double stranded target cannot reanneal and,

– excess probe can easily be removed after the reaction.

A major drawback is the immobilization step itself. When crude biological samples are contacted with the support, non-nucleic acid compounds are bound in addition to the target. This often causes background problems. Another limitation of this approach is that the reactions are carried out in a non-homogeneous medium, and consequently the hybridization reaction is slowered due to accessibility problems for the reacting molecules [2].

Both the primary collision between the two DNA strands and the subsequent winding process obviously proceed faster in solution.

Thus it is preferable to perform the hybridization assay in solution. However, the labelled probe is

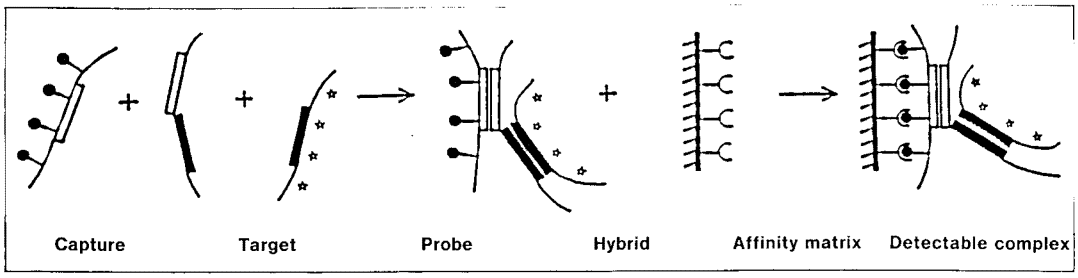

Fig. 1. – The principle of the affinity based hybrid collection method [3].

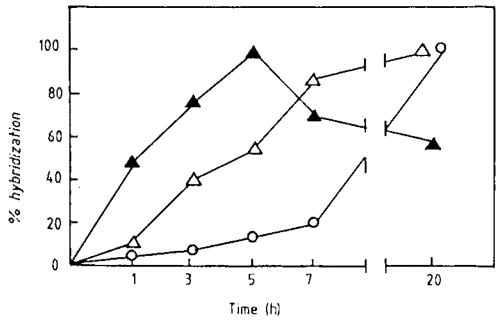

Fig. 2. – Comparison of the reaction rate in sandwich hybridization when the capture probe is immobilized on a filter (0), immobilized on microparticles (△), or immobilized after the hybridization reaction with the help of an affinity label (◭) [4].

generally present in more than 1 000 fold excess over the target. The separation of such an excess of free probe from the hybrids is a difficult task.

Affinity based hybrid collection

To advantage of the fractionation power of assays with immobilized targets, but to retain the advantages of solution hybridization, we developed the affinity based hybrid collection method (ABC) [3]. In this assay two non-overlapping probes are used to recognize the target nucleic acid. The use of two probes increases the specificity since two regions on the target must be recognized simultaneously in order to form the sandwich hybrid. The principle of the method is shown in Figure 1.

One of the probes (the capturing probe) is chemically derivatized to contain a moiety with high affinity for another chemical group. We call these compounds affinity pairs. A good pair is e.g. avidin and biotin. The other probe (the detector probe) is labelled with a detectable moiety such as a radioisotope, a fluorochrome or an enzyme. The sandwich hybrid formed is isolated with the help of the affinity group on the capturing probe (Fig. 1). Th specificity of the affinity pair allows at least an 10.000 fold purification of hybrids from free detector probe. This is a prequisite for the detection of attomole levels of target.

The kinetic advantage of the hybridization in solution is demonstrated in Figure 2. Here the reaction rate using affinity labelled capture DNA is compared to that of preimmobilized capture. The data is from reference 4.

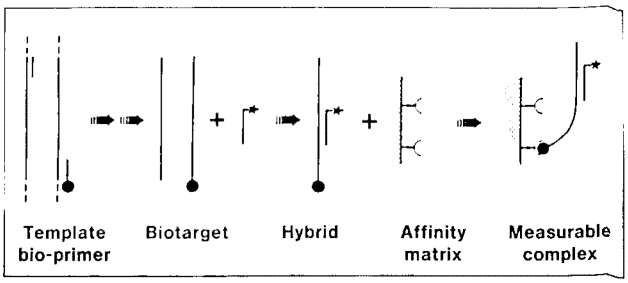

Fig. 3. – The principle of the PCR product collection method [8].

The ABC principle is used in the AffiProbe HPV identification kit (Orion Biotechnology) for the detection and typing of human papillomavirus. In this test biotinylated capturing and ^{35}S labelled detector probes are allowed to hybridize to DNA in a crude cell lysate for 3 hours. The hybrids are then captured in microtiter wells coated with streptavidin.

The test quantifies the amount of target in the sample over a range from $5 \times 10^5 - 5 \times 10^8$ (1 attomole - 1 femtomole) in a one day assay [5].

Even if attomole sensitivity is useful in HPV typing, it is not sufficient for all applications. So far the most frequently used non-radioactive labels do not allow a significant improvement in the sensitivity of detection compared to radioisotopes.

Quantification of PCR-products

A promising approach to solve the sensivity problem in hybridization assays is to increase the number of target molecules in vitro before the detection. This can be done using the polymerase chain reaction (PCR) [6]. By 20 cycles of PCR 1.000 molecules of target DNA can be amplified to about 10^8 molecules. A number of available detection methods [7] can consequently be used with a very high sensitivity in regard to the original amount of target. However, it should be noted that PCR frequently amplifies unspecific DNA fragments. To reliably identify and quantify the specific PCR product, it should be detected by hybridization with a probe recognizing the nucleotide sequence internal to the two primers. In quantifying the PCR product the method of choice depends partly on the same factors as those presented above for direct detection of polynucleotide sequences.

We have modified the principle of affinity collection for use in PCR as shown in Figure 3. Here the affinity moiety is attached to the 5' end of the PCR primer. Thus all molecules synthetized during the PCR process will carry e.g. a biotin residue. Using an oligonucleotide probe, hybridization in solution, and affinity capture of the formed hybrids, the PCR product is conveniently and accurately quantified in less than 1 hour [8].

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