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Fast quantification of nucleic acid hybrids by affinity-based hybrid collection

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ABSTRACT

A hybridization technique for the quantification of nucleic acids is described. In the method a probe pair is allowed to form hybrids with the target nucleic acid in solution. One of the probes has been modified with an affinity label, by which the formed hybrids can be isolated after the reaction. Strepta-vidin-agarose was used to capture hybrids containing biotinyla-ted DNA. The hybrids were measured using radioiodine as label on the second probe. The rate of the hybridization reaction in so-lution is fast, allowing the whole procedure to be carried out in 3 h. The method is quantitative with a detection limit of 4 x 10^5 molecules (0.67 attomoles) target DNA. The test is insensi-tive to impurities in biological samples, which are analyzed without purification of the target DNA. Non-isotopic measurement of the hybrids can also be applied. In this case the hybrids are bound to microtitration wells and detected spectrophotometri-cally by peroxidase-catalyzed colour development.

INTRODUCTION

In the hybridization techniques most commonly used today the nucleic acids to be analyzed are fixed on a filter. The se-quences of interest are then detected after hybridization to a labelled probe. A tedious procedure to purify and immobilize the target nucleic acid is usually required in these methods, but it is easy to separate hybridized from free probe. The sandwich hybridization technique developed in our laboratory (1) is more convenient to use for analysis of biological samples than direct hybridization methods (2-4). In sandwich hybridization target DNA in solution is allowed to anneal to the labelled probe and to immobilized capture DNA. Usually nitrocellulose filters have been the solid support (1-4), but other carriers such as poly-propylene and agarose (5) or Sephacryl (6) can also be used.

The annealing reaction between complementary nucleic acid strands is slow because the molar concentrations of the reac-

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tants are usually extremely low (7). When one of the nucleic acid strands is immobilized the reaction rate is further decreased (8). This problem is especially pronounced in sandwich hybridization techniques, where the target DNA is the rate-limiting component. Thus the reaction rate can be increased only to some extent by raising the concentration of the probe DNA (1). Provided that the specific hybrids can be isolated or detected after the reaction, it is advantageous that all components of the hybridization reaction are present in solution. However, complete separation of hybridized probe molecules from free probe requires complicated isolation systems, often including enzymatic digestions (9-11).

Chemical groups such as biotin (12-15) or haptens (16-17) can be introduced into nucleic acid probes. The modified probes can be detected by non-radioactive methods after hybridization to immobilized DNA (16,18,19). Chemical groups introduced into nucleic acid probes have also been used as affinity labels to isolate sequences of interest by hybridization followed by affinity chromatography on solid matrices. Biotinylated DNA in conjunction with avidin matrices (12,20,21), mercurated DNA with thiol matrices (22,23) and poly(A)-tailed RNA with an oligo(dT) matrice (24) are examples of affinity pairs used for isolation of specific DNA fragments.

Here we describe the development of a nucleic acid hybridization technique, in which the advantages of hybridization in solution, sandwich hybridization and affinity labelled hybridization probes are utilized.

MATERIALS AND METHODS

Probe and target DNA

The recombinant clones used as probe and capture DNA reagents were derived from the plasmid pBR 322. The phages mKTH 1301 and mKTH 1302 contain the 1.4 kb PstI-SalI fragment of pBR 322 cloned into the respective sites of M 13 mp10 and mp11. In the plasmid pKTH 1300 the 1.4 kb PstI-SalI fragment of pBR 322 has been deleted and substituted with the 1.4 kb kanamycin resistance gene of the plasmid pUC-4K (Korpela, K. et al. to be published). Plasmid pBR 322 linearized with EcoRI served as target DNA.

Labelling of probe and capture DNA

Plasmid pKTH 1300 DNA was labelled by nick translation (25) with ^{125}I-dCTP (IM 5103; Amersham, U.K.) to specific activities of about 10^8 cpm/µg, which yielded a mixture of fragments 4.3 0.5 kb in length. Single-stranded mKTH 1301 DNA was iodinated chemically (Na^{125}I; IMS 30, Amersham, U.K.) by a modification of (26) to 10^8 cpm/µg and a fragment size of 0.6-0.4 bp (Laaksonen, M. et al. unpublished). Biotin was introduced into pKTH 1300, mKTH 1301 and 1302 using photobiotin^{TM} (BRESA, Adelaide, South Australia) (14) and into pKTH 1300 by nick translation (12) with biotin-11-dUTP (Bethesda Research Laboratories, Maryland, U.S.A.). The plasmid pKTH 1300 was modified by a sulfonation reaction (27) using reagents from Orgenics Ltd., Yavne, Israel.

The hybridization reaction

Denaturated target DNA was allowed to hybridize to the labelled capture and probe DNA in solution. Usually 5 x 10^9 molecules (24 ng) each of the biotinylated capture mKTH 1301 and 1302 DNA and 2 x 10^8 molecules (10^5 cpm, 0.94 ng) ^{125}I- labelled pKTH 1300 probe DNA were used per reaction. Alternatively 4 x 10^9 molecules (19 ng) sulfone-modified pKTH 1300 served as capture DNA and 10^{10} molecules (47 ng) each of biotin-labelled mKTH 1301 and 1302 as probe DNA. The hybridization reactions were carried out in 40 µl of a solution containing 0.6 M sodium chloride, 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 0.1 % sodium dodecyl sulphate (SDS) at 65°C in Eppendorf-tubes under a layer of viscous paraffin to avoid evaporation. In some cases 2.5-10 % polyethylene glycol (PEG 6000) was included in the reaction mixture.

Collection and measurement of the hybrids

After hybridization with ^{125}I-labelled probe and biotinylated capture DNA a batch procedure was used for collection of the hybrids on a streptavidin-agarose matrice. The samples were diluted to 100 µl with a solution of 1 M sodium chloride, 10 mM sodium phosphate, pH 7.5, and 1 mM EDTA. 100 µl of a 50 % suspension of streptavidin-agarose (Bethesda Research Laboratories, Maryland, U.S.A.) in the buffered 1 M sodium chloride solution was added and the tubes were incubated for 15 min at 37°C in a rotating mixer. The supernatant was discarded after brief centrifugation, and the agarose was washed with 1 ml of the buffered

1 M sodium chloride solution for 5 min in the rotating mixer. Then the agarose was washed twice with 1 ml of a solution containing 15 mM sodium chloride, 1.5 mM sodium citrate, 0.2 % SDS by keeping the tubes for 1 min at 55°C and for additional 5 min in the rotating mixer at 37°C. The radioactivity of the hybrids bound to the streptavidin-agarose was measured in a gamma-counter.

When using biotin-labelled probe DNA and sulfone-modified DNA as capturing reagent the hybrids were collected in polystyrene microtitration wells precoated with a monoclonal antibody against sulfone-modified DNA (Orgenics Ltd., Yavne, Israel). The wells were coated with purified IgG at 10 µg/ml in 10 mM sodium carbonate buffer; pH 9.6, over night at 4°C. After washing three times with 0.1 % Tween 20 in 0.15 M sodium chloride, 20 mM sodium phosphate; pH 7.5 (PBS) unspecific binding sites of the wells were blocked for 30 min at 37°C with 0.2 mg/ml denaturated herring sperm DNA in PBS. 120 µl dilution buffer was added to the hybridization mixture to yield a final concentration of 1 % bovine serum albumin (BSA), 0.15 M sodium chloride, 10 mM sodium phosphate; pH 7.5, and 0.1 % Tween 20 and transferred to the coated microtitration wells. The microtitration plates were incubated for 3 h at 37°C with gentle shaking and the wells were washed three times as above. Then 160 µl of a horseradish peroxidase-streptavidin conjugate (Amersham, U.K.) diluted 1:2000 in 1 % BSA, 0.1 % Tween 20 in PBS was added, and the plates were incubated for 45 min at 37°C with gentle shaking. After four washes as above, 160 µl of substrate solution containing 0.46 mg/ml o-phenylene-diamine, 0.01 % H_2O_2 in 0.1 M sodium acetate buffer, pH 5.0, was added. The reaction was stopped after 15 min at 22°C by addition of 50 µl 2N H_2SO_4, and the absorbance of the coloured product in the wells was measured with a spectrophotometer at 492 nm.

RESULTS AND DISCUSSION

The principle of the method

In the method described here the target nucleic acid is allowed to hybridize simultaneously to two different DNA fragments homologous to the target DNA, but non-homologous to each other. During the reaction, which is carried out in solution, hybrids of the target DNA are formed with both molecules. One of

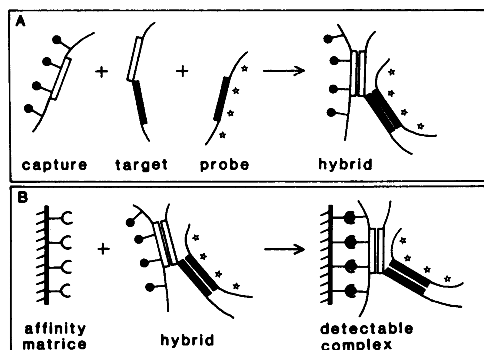

Figure 1.
Principle of the affinity-based hybrid collection procedure.
(A) The target nucleic acid is in solution allowed to form hybrids with a capturing probe modified with an affinity label and a probe carrying a detectable label.
(B) After the reaction the specific hybrids formed are collected on a solid affinity matrice with the aid of the label on the capturing probe and quantified by the label on the second probe.

the fragments is tagged with an affinity label (the capture DNA) enabling collection of the specific hybrids on a solid affinity matrice after the reaction. The second fragment (the probe DNA) carries a different label by which the isolated hybrids are quantified (Figure 1). We used either biotinylated DNA or DNA modified with a hapten as capturing reagent and streptavidin or antibodies against the hapten on the affinity matrice, respectively. The hybrids were measured isotopically or using an enzymatic detection system.

The optimal procedure

The collection of hybrids from the reaction mixture is the crucial step in the method described here. Batch and column procedures for collection of hybrids with biotinylated capture DNA on a streptavidin-agarose matrice were compared. The best results were obtained with a batch procedure, where the hybrids were allowed to bind to the streptavidin for 15 min at 37°C.

The lowest background from unspecific binding of DNA to the agarose was seen using streptavidin-agarose from BRL and by washing the agarose after binding of the hybrids with high salt, followed by the stringent wash as described in "Materials and Methods". This resulted in background levels of 100-200 cpm, but

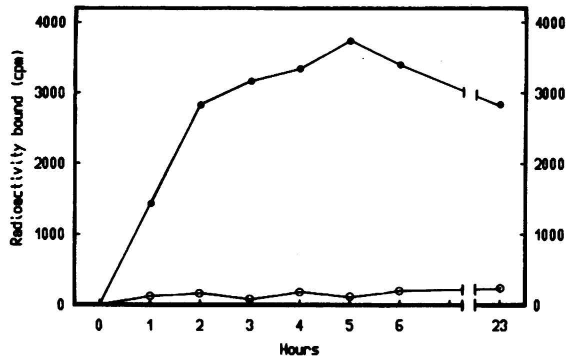

Figure 2.
Kinetics of the hybridization reaction. Target DNA (5 x 10^7 molecules) was allowed to hybridize to biotinylated mKTH 1301 and 1302 DNA (5 x 10^9 molecules of each) and ^{125}I-labelled plasmid pKTH 1300 DNA (2 x 10^8 molecules = 10^5 cpm) for different time periods. The radioactivity of hybrids collected on streptavidin-agarose (●——●) and background from control hybridizations without target DNA (o——o) are shown. Each point represents the mean cpm-value of triplicates.

also in some loss of specific signals.

Equal signals from the hybrids were obtained using biotinylated M 13 DNA as capturing reagent and ^{125}I-labelled plasmid DNA as detectable probe or using biotinylated plasmid DNA in conjunction with ^{125}I-labelled M 13 probe DNA. Despite higher biotinylation levels achieved by nick translation (unpublished observation, 12,14) the signals obtained using photobiotinylated plasmid DNA are similar to those of capture molecules biotinylated by nick translation. In the following we used photobiotinylated M 13 DNA and nick translated ^{125}I-labelled plasmid DNA.

With the M 13 capture reagent there is an option to use either one M 13 clone or, alternatively, two clones with the same insert in opposite polarity. In the first case only one strand of the target will be detected, in the second case the clones will also hybridize to each other reducing the concentration of available capture reagent. The use of both polarities gave 10-20% higher signals than with one polarity alone.

Kinetics of the reaction

The major advantage of carrying out the hybridization reaction in solution is the considerably faster reaction rate than in mixed-phase hybridizations. Figure 2 illustrates the rate of

Table 1
Effect of polyethylene glycol (PEG) on the reaction rate.

% PEG	Radioactivity bound (cpm)[1) Target DNA]	Control
0	1628	119
2.5	2256	130
5.0	2856	295
7.5	4747	536
10	24137	20138

Target DNA (5×10^7 molecules) was hybridized to biotinylated mKTH 1301 and mKTH 1302 DNA (5×10^9 molecules of each) and ^{125}I-labelled plasmid pKTH 1300 DNA (2×10^8 molecules = 10^5 cpm) for 1 h in the presence of varying concentrations of PEG. Control reactions without target DNA were included. 1) Mean value of triplicates.

the solution hybridization reaction when the biotinylated capture DNA was present in 100-fold and the iodinated probe DNA in 4-fold molar excess over the target DNA. The reaction was maximal in 5 h and 72 % of this level was achieved in 2 h. In mixed-phase sandwich hybridization less than 10 % of the maximal signal is obtained in 2 h (1). As can be seen in Figure 2 the signal decreased after hybridization for 23 h. It is possible that upon prolonged incubation some of the target DNA reanneals, followed by branch migration and displacement of the labelled probe.

At the target DNA concentrations used in the experiment shown in Figure 2, and more significantly at lower concentrations, the target DNA is the rate-limiting component of the reaction. The effective concentration of target DNA can be increased by adding inert macromolecules, which act as volume excluders, such as polyethylene glycol or dextran polymers (28), to the reaction mixture. The results in Table 1 show the effect of adding PEG to the hybridization mixture. The signal obtained in a 1 h reaction was clearly increased, but some increase in backgrounds was also observed. The highest signal to noise ratio was obtained with 2.5 % PEG. 10 % PEG caused precipitation or unspecific binding of the probe DNA to the agarose matrice.

Sensitivity

A standard curve obtained with the affinity-based hybrid

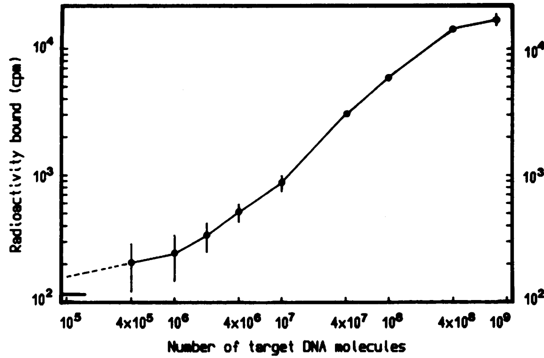

Figure 3.
Standard curve of the affinity-based hybrid collection procedure. The radioactivity in recovered hybrids after hybridization of biotinylated mKTH 1301 and 1302 DNA (5 x 10^9 molecules of each) and ^{125}I-labelled plasmid pKTH 1300 (2 x 10^8 molecules 10^5 cpm) with increasing amounts (4 x10^5 - 10^9 molecules; 1.9 pg-4.7 ng) of target DNA is shown. The reaction time was 3 h. Each point represents the mean cpm-value of four determinations and the vertical bars mark the SD of the signals. The background radioactivity of control reactions without target DNA is indicated by the horisontal bar.

collection procedure is presented in Figure 3. There is a linear relationship between the amount of target DNA and the radioactivity in the hybrids bound to the streptavidine-agarose matrice. The lowest amount of target DNA detected was 4 x 10^5 molecules equalling 0.67 x 10^{-18} moles or 1.7 pg target sequences. At this concentration 200 cpm of ^{125}I was bound to the hybrids. This radioactivity corresponds to 4 x 10^5 molecules of the probe DNA (specific activity 10^8 cpm/µg), indicating that the reaction has gone essentially to completion. (Possible ladder formation of the double-stranded probe DNA has not been taken into account in this calculation.) Consequently, the sensitivity of the method can be increased only by using probes of higher specific activity (19) or by amplifying the target DNA (29).

We have not been able to drive the mixed-phase sandwich hybridization reaction to completion because the reanneling of target DNA in solution is faster than annealing to the immobilized capture DNA. For the same reason mixed-phase sandwich hybridization cannot be enhanced by volume excluders (1).

Table 2.
Effect of crude biological material on the hybridization signal

Additive[1)]	Amount	Relative signal (%)[2)]
None	-	100
E.coli HB101	2 x 10^7 cells	93
"	10^7 "	99
Vero	10^6 "	74
"	5 x 10^5 "	92
"	10^5 "	94
Human serum	20 µl	30
"	10 "	48
"	5 "	54
"	2.5 "	73

Target DNA (5 x 10^7 molecules) was hybridized to capture and probe DNA in the prescence of crude biological material for 3 h. 4 % PEG was included in the reactions with B-vero cells and serum. 1) Before addition to the reaction the E.coli HB101 cells were treated with 1 mg/ml lysozyme for 30 min at 37°C and with 1% SDS for 5 min at 100°C, the Vero cells were lysed with 1 % SDS, sheared mechanically using a hypodermic needle, and kept for 5 min at 100°C and the serum samples were treated with 1 mg/ml proteinase K and 1 % SDS for 30 min at 37°C followed by 5 min at 100°C. 2) Per cent-values of the signal compared to that from a hybridization with 5 x 10^7 molecules pure target DNA.

Analysis of biological samples

Bacterial (E. coli HB101) and mammalian (Vero) cells and human serum were chosen to test the applicability of the test to crude biological samples. After simple pretreatment (see Table 2) varying amounts of the samples were added to the hybridization reaction together with a constant amount of target DNA. The signals obtained in comparison with signals from reactions with pure target DNA are shown in Table 2. Sample DNA present in at least 2 x10^7 E.coli cells and 5 x 10^5 Vero cells can be quantified. Furthermore the method allows identification of DNA of interest in 10^6 mammalian cells and in 20 ul serum (equalling 50 % serum during hybridization). None of the crude samples tested caused any background above the level obtained with pure DNA. Thus the major advantage of mixed-phase sandwich hybridization, the possibility of analyzing unpurified biological material in long sample series, has been retained.

In the experiments presented in Table 2 the reaction volume was 40 µl. If the concentrations of the capture and probe DNA

are kept constant the reaction volume can be increased at least 4-fold without loss of sensitivity (data not shown), which makes it possible to analyze larger biological samples.

Enzymatic hybrid detection

Hybridization reactions can be drived to increased reaction rates using high probe concentrations. However, with radioisotopes background problems and the use of radioactivity itself set a limit to this approach. A modification of the method described above was designed, in which a non-radioactive probe and the routine laboratory procedures of enzyme immunoassays are used.

Sulfone-modified plasmid DNA served as the capture reagent and biotinylated M13 DNA as the detectable probe. After hybridization to target DNA in solution the hybrids were collected in microtitration wells precoated with antibodies against sulfone-modified DNA. The bound hybrids were detected with a streptavidin-horseradish peroxidase conjugate catalyzing the formation of a coloured product, which was measured in a spectrophotometer.

A standard curve of a 1.5 h hybridization reaction is shown in Figure 4. The measured absorbance-values are proportional to the amount of target DNA present in the reaction. The detection

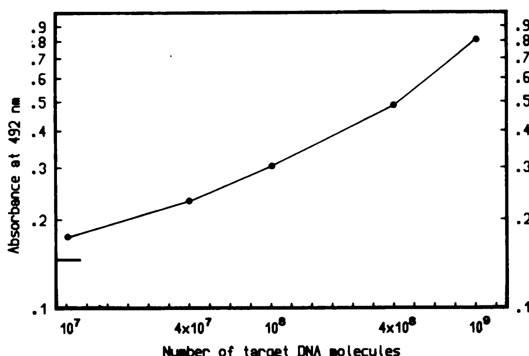

Figure 4.
Standard curve of the affinity-based hybrid collection procedure with enzymatic detection. Increasing amounts of target DNA (10^7-10^9 molecules; 47 pg-4.7 ng) were hybridized to sulfone-modified plasmid pKTH 1300 DNA (4 x 10^9 molecules) as capturing reagent and biotin-labelled mKTH 1301 and 1302 (10^{10} molecules of each) as probe DNA for 1.5 h. The hybrids were collected and measured in microtitration wells. Each point is the mean absorbance reading of duplicate assays. The horizontal bar indicates the background from control reactions without target DNA.

sensitivity was 10^7 molecules (16×10^{-18} moles, 47 pg) target DNA. This is as expected since the sensitivity using enzymatic detection systems is usually between 1/10 and 1/100 of that obtained with radioisotopes (16,18).

The non-radioactive detection system resembling conventional enzyme immunoassays, makes it possible to use already existing laboratory equipment. The results of the test obtained from an automatic spectrophotometric reader are numeric, rather than visual, which fascilitates quantification. Thus the affinity-based hybrid collection procedure is suitable for use in diagnostic medicine and other applications.

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