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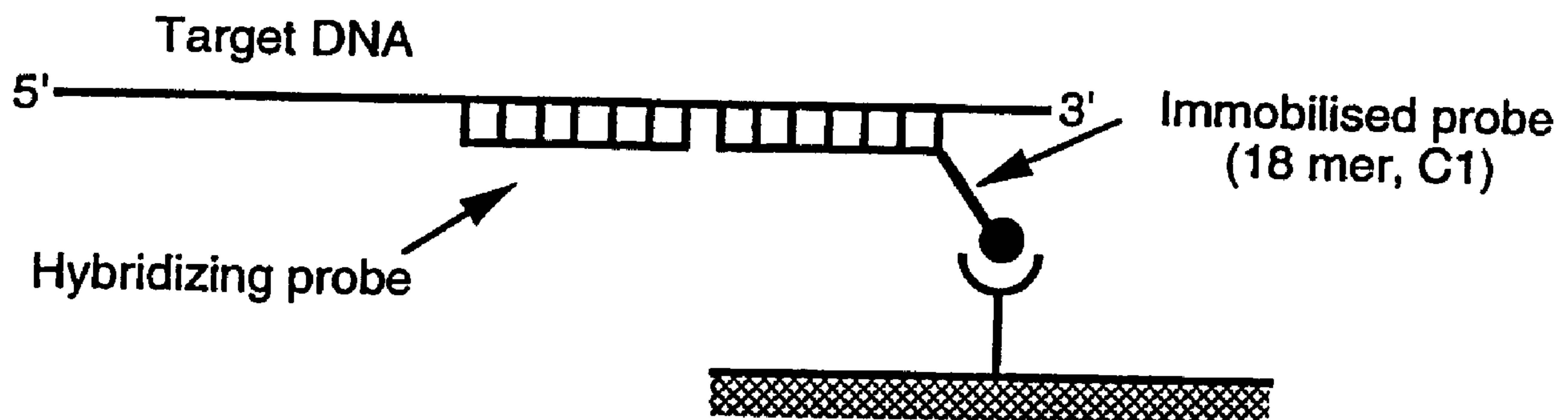
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(54) Titre : UTILISATION D'OLIGONUCLEOTIDES MODULAIRES COMME SONDAS OU AMORCES DANS DES
DOSAGES A BASE D'ACIDE NUCLEIQUE

(54) Title: THE USE OF MODULAR OLIGONUCLEOTIDES AS PROBES OR PRIMERS IN NUCLEIC ACID BASED
ASSAY



(57) Abrégé/Abstract:

The invention provides a method of improving the binding of a series of consecutive nucleotide bases to a complementary target nucleic acid molecule in a sample, wherein said method comprises at least the step or steps of binding a complementary modular oligonucleotide of at least two parts (modules) including said nucleotide bases to adjacent stretches of said target nucleic acid molecule in said sample, especially methods of detection/isolation and method in which the modular oligonucleotide is a primer, modular oligonucleotides themselves and their use in methods of the invention.

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(54) Title: THE USE OF MODULAR OLIGONUCLEOTIDES AS PROBES OR PRIMERS IN NUCLEIC ACID BASED ASSAY (57) Abstract The invention provides a method of improving the binding of a series of consecutive nucleotide bases to a complementary target nucleic acid molecule in a sample, wherein said method comprises at least the step or steps of binding a complementary modular oligonucleotide of at least two parts (modules) including said nucleotide bases to adjacent stretches of said target nucleic acid molecule in said sample, especially methods of detection/isolation and method in which the modular oligonucleotide is a primer, modular oligonucleotides themselves and their use in methods of the invention.		

THE USE OF MODULAR OLIGONUCLEOTIDES AS PROBES OR PRIMERS IN NUCLEIC ACID BASED ASSAY

The present invention relates to a method of improving the binding of a series of consecutive nucleotide bases to a complementary nucleic acid molecule, especially for use in improving the binding of capture oligonucleotides, modular oligonucleotides and kits for performing methods of the invention.

The binding of complementary nucleotide bases to one another represents one of the most significant and fundamental findings in science this century and heralded the rapid development of the field of biochemistry. Whilst allowing an understanding of the mechanisms underlying the continuation of life, the discovery has also provided the basis for the development of valuable molecular biological tools.

The isolation and sequencing of naturally occurring nucleic acid molecules is a common goal for molecular biologists. The use of complementary oligonucleotides to isolate nucleic acid molecules is commonplace. Similarly, complementary oligonucleotides are frequently used to bind single-stranded nucleic acid molecules and act as primers for extension reactions to produce complementary strands to the template and forms the basis of such experimental procedures as polymerase chain reaction (PCR) and sequencing reactions.

However, the specificity of binding of oligonucleotides to template or target DNA depends on a number of parameters any one of which may result in poor efficiency of binding and consequently poor experimental results. The specificity of the interaction may conveniently be determined by the assessment of T_m , the temperature at which duplexes dissociate. This is however also dependent on other parameters, for example the buffer in which the reaction is performed. For a particular experimental system, T_m will be affected by various factors including the extent of complementarity, the sequence of the target and/or oligonucleotide,

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derivatization of the oligonucleotide and length of the oligonucleotide. The binding of oligonucleotides may therefore be improved, as evidenced by an increased T_m under the same experimental conditions, by altering these parameters. However, the variation which may be achieved by altering these parameters is limited. There therefore exists a need for further methods which will improve the binding of oligonucleotides to target DNA.

Surprisingly, it has now been found that modular probes or primers composed of at least two modules (oligonucleotides) which bind to adjacent regions of target DNA exhibit improved binding relative to a single oligonucleotide spanning the same length as the separate modules. For example, it has been found that two adjacent 18-mer oligonucleotides bind more efficiently to target DNA than the composite 36-mer oligonucleotide.

The use of primers composed of adjacent modules for sequencing purposes has been described previously (Kotler et al., 1993, Proc. Natl. Acad. Sci. USA, 90, p4241-4245; Kieleczawa et al., 1992, Science, 258, p1787-1791 and Szybalski, 1990, Gene, 90, p177-178). However, in these cases the modular primers were used to replace longer primers such that libraries of all sequences of the shorter primers could realistically be pre-synthesized as they had fewer possible sequence permutations than longer primers. In all cases, the modular primers were only shown to have, in sequencing reactions under the same conditions, efficacy as good as the longer primers. In contrast, in the present invention, surprisingly, even better binding is achieved, when a single oligonucleotide is split into separate components. Furthermore, the previous work indicates that the effect of modular primers may only be achieved if the modules do not have a single (or more) base(s) between them when bound to the template. For improved binding as described herein, no such restriction is applicable although even better binding is observed when no gaps exist between the

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modules.

Lin et al. (Lin et al., 1989, Biochemistry, 28, p1054-1061) describes that a cooperative effect, probably due to base-stacking, occurs between adjacently bound oligonucleotides which increases the T_m . This however is compared only to the binding of one of the modules of the modular primer and not a composite primer of the full length of the modular primer. The findings presented here thus present a considerable advance over the prior art and have many applications for instances in which improved binding of an oligonucleotide to a target nucleic acid molecule is required.

Thus, viewed from one aspect, the present invention provides a method of improving the binding of a series of consecutive nucleotide bases to a complementary target nucleic acid molecule in a sample, wherein said method comprises at least the step or steps of binding a complementary modular oligonucleotide of at least two parts including said nucleotide bases to adjacent stretches of said target nucleic acid molecule in said sample, wherein said modular oligonucleotide exhibits improved binding relative to a single oligonucleotide complementary to the region of the target molecule spanned by the modular oligonucleotide.

Alternatively viewed, the present invention provides a method of binding a series of consecutive nucleotide bases to a complementary target nucleic acid molecule in a sample, wherein said method comprises at least the step or steps of binding a complementary modular oligonucleotide of at least three parts including said nucleotide bases to adjacent stretches of said target nucleic acid molecule in said sample.

As used herein, the term "improving" with respect to binding is intended to indicate increased specificity, stability or ability to bind to target nucleic acid molecules. "Binding" may be determined according to any method known in the art (for example as described herein)

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and will, as will be clear to the skilled addressee, be dependent on establishing appropriate buffer, temperature and other conditions. Binding of the modular oligonucleotide may be performed by binding all the parts thereof simultaneously or alternatively, sequential steps involving binding one or more of the modules at each step may be performed. "Complementary" as used herein is intended to encompass any series of consecutive nucleotide bases, oligonucleotide or target/template nucleic acid, as appropriate, which is complementary to the nucleotide sequence of the nucleic acid molecule in question, or its corresponding RNA, DNA, or nucleic acid analog, peptide nucleic acid (PNA). Modules of the modular oligonucleotide may be formed as a composite of the different nucleic acid molecules, e.g. DNA and PNA. Alternatively, individual modules may be composed exclusively of RNA, DNA or PNA, but different modules within the modular probe may be of a different nucleic acid. Thus, for example, a PNA module may be used as a capture probe whereas adjacent modules may be composed of DNA such that extension procedures (e.g. RT-PCR, DNA sequencing etc.) may be performed using the DNA module. Complementarity of the nucleic acid molecules includes within its scope non-absolute complementarity in which some mismatching may occur, although the "complementary" nucleic acids, or oligonucleotides or series of nucleotides, as appropriate, bind to one another under conditions of high stringency. Such oligonucleotides are those which bind under non-stringent conditions (e.g. 6xSSC/50% formamide at room temperature) and washed under conditions of high stringency (e.g. 2xSSC, 65°C), wherein SSC=0.15M NaCl, 0.015M sodium citrate, pH 7.2.

"Nucleic acid molecule" is intended to cover inter alia RNA, mRNA, DNA, cDNA e.g. from retroviral RNA, genomic DNA, mitochondrial DNA etc. and PNA. The DNA may be single or double stranded. When double stranded DNA is used, appropriate procedures may be necessary to allow

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binding of the modular oligonucleotide, for example by heating to disrupt the structure to the single stranded form. "Target" nucleic acid includes molecules which are detected or isolated according to methods of the invention in addition to molecules which serve as a template for certain molecular reactions, for example, amplification, sequencing or transcription for the preparation of further distinct molecules. Nucleotide bases or oligonucleotides which bind to the target nucleic acid molecule may be modified or derivatized, providing they retain the ability to fulfill the complementarity requirements described above. For example, methylated, ethylated or carboxylated bases or other such modified or unusual bases may be used. Alternatively, the nucleic acid backbone may be modified, e.g. PNA units. Alternatively the base may carry a label, for example a hapten such as biotin or a dye. "Oligonucleotides" encompass any piece of DNA (or RNA after reverse transcription), RNA or PNA and extends also to the use of chimers of RNA, DNA and/or PNA.

"Modular oligonucleotide" refers to the primer/probe oligonucleotide which is composed of more than one part. Each part is an oligonucleotide which is referred to as a module of the whole. "Adjacent" as used herein is intended to signify non-overlapping regions of the nucleic acid molecule which lie close to one another, for example are less than 100 or 50 nucleotide bases apart, preferably 10 bases apart, especially preferably less than 2 bases apart, and most preferably without any bases in between, i.e. directly adjacent. Thus, the "single oligonucleotide" referred to above which comprises the modular oligonucleotide may include more nucleotides than the sum of the nucleotide bases in all parts of the modular oligonucleotide as the bases complementary to the region between the binding site of each module of the modular oligonucleotide will also be included in instances in which the modules, when bound,

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are not directly adjacent.

The method of the invention described herein may be used for any application in which improved binding of nucleotide bases, preferably in the form of an oligonucleotide, to a target nucleic acid molecule is required. Whilst not wishing to be bound by theory, it appears that the use of modular oligonucleotides allows the disruption of tertiary structures of nucleic acid molecules which are present not only in tRNA but also in other nucleic acid molecules. Such tertiary structures do not appear to be as effectively disrupted using longer oligonucleotides in which the parts of the modular oligonucleotide are synthesized together as a single molecule. Thus, applications which require improved binding to areas of nucleic acid molecules with tertiary structure which would prevent or impair binding of an oligonucleotide to this region, will benefit from this invention. The present invention therefore extends to, but is not limited to, applications in which the modular oligonucleotide serves as a primer in methods which involve replication, amplification, transcription, reverse transcription and/or sequencing, or in which the modular oligonucleotide serves as a probe for detection and/or capture or isolation of target nucleic acid molecules. It will be appreciated that in appropriate circumstances modular oligonucleotides may serve both of the aforementioned functions, e.g. by serving both as a primer and also as a capture/detection probe for the nucleic acid products e.g. amplified DNA, thus produced.

In the case of sequencing reactions, it may be found that a primer, regardless of its length is unable to provide the required reaction products. Such a problem may be overcome by the use of a modular primer as an alternative to a composite primer. This may be achieved by simply including a second primer into the sequencing reaction in addition to the first primer, which binds at the front or rear of the first

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(sequencing) primer, as appropriate, and allows improved binding of the first primer to the template thereby causing or improving an appropriate sequencing reaction. Such improved binding, according to the definition of this invention, would not be observed if the second primer were simply ligated to the terminal end of the first sequencing primer. Alternatively, if the sequencing primer which gives a poor result is sufficiently long, a modular primer in which the sequencing primer is divided into at least two parts may be employed. If one of the modules of the primer is immobilized on a solid support, sequencing reactions may be performed directly on the support (Sanger T7 DNA polymerase sequencing) or used in cycle sequencing (Taq DNA polymerase).

In a similar way, the use of a modular primer may improve or cause replication, amplification, reverse transcription or transcription of a template nucleic acid molecule in a superior manner to that using a single primer composed of the separate modules.

The introduction of modules which bind adjacent to the primer in such reactions may enhance the reactions therefore increasing overall sensitivity. As indicated earlier, this invention may result from the disruption of tertiary structures in nucleic acid molecules. Tertiary structures have been reported to be of critical importance in the Q-beta replicase reaction (Kramer and Lizardi, 1989, Nature, 339, p.401-402). Thus, in a preferred aspect the invention provides a method of replication, amplification, transcription, reverse transcription and/or sequencing a target nucleic acid molecule in a sample, wherein said method comprises the binding of a complementary modular oligonucleotide as defined herein as a primer in the method.

Preferred applications of the present invention include the detection and/or capture of target nucleic acid molecules in which the binding of a probe to target

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nucleic acid is improved by the use of a modular probe with at least two parts. Such an application may be, for example, in Southern blot analyses for detecting target nucleic acid molecules to which a composite probe does not bind effectively. As used herein a composite is intended to mean that oligonucleotide which would result from the synthesis of appropriate modules as a continuous oligonucleotide, including the insertion of any necessary nucleotide bases complementary to the bases of the target nucleic acid between modules which are not directly adjacent. This may be improved by the use of a modular probe. Thus for example if a ten-mer oligonucleotide does not bind effectively to a target molecule, this may be replaced by two five-mer oligonucleotides, or a five-mer oligonucleotide may be added. In this way, the binding of the modular probes (comprising in total, in this example, 10 or 15 nucleotides) is improved relative to the binding of a composite probe of the 10 or 15 nucleotides (or more if the parts of the probe are not directly adjacent once bound to the target), respectively.

This method has been found to be highly effective for the detection and isolation or capture of target DNA in solution. In this method, a modular probe composed of at least two modules is employed. One module of the modular probe is the capture or detection module (or oligonucleotide). The further modules (modulators) assist by improving the binding of the capture module to the target molecule.

Thus, viewed from a further aspect, the present invention provides a method of detecting and/or isolating a target nucleic acid molecule in a sample, wherein said method comprises at least the step or steps of binding a complementary modular oligonucleotide of at least two parts to adjacent stretches of said target nucleic acid molecule in said sample.

Preferably for this method, if the capture or

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detection module and the modulator module(s) were to form a single oligonucleotide probe, the binding efficiency would be decreased relative to the binding of the capture or detection module to the target in the presence of the free modulator modules.

Thus, viewed from a yet further aspect, the present invention provides a method of detecting and/or isolating a target nucleic acid molecule in a sample, wherein said method comprises at least the step or steps of binding a complementary modular oligonucleotide of at least two parts to adjacent stretches of said target nucleic acid molecule in said sample, wherein said modular oligonucleotide exhibits improved binding relative to a single oligonucleotide complementary to the region of the target molecule spanned by the modular oligonucleotide.

"Isolating" as used herein is intended to encompass the capture of target nucleic acid, even if this is not removed from the sample in which it is present, ie. physical separation or purification is not necessarily performed. Such methods involve the "capture" of the target from the sample in which it is contained by binding an oligonucleotide to it, thus effectively isolating it from other DNA molecules present in the sample. In methods of isolation the capture module will thus function also as the isolation module, allowing target molecules bound to it to be isolated.

Also provided according to the invention is a method of replication, amplification, transcription and/or reverse transcription of a target nucleic acid molecule in a sample, wherein said method comprises at least the step or steps of binding a complementary modular oligonucleotide of at least two parts to adjacent stretches of said target nucleic acid molecule in said sample.

Preferably, when isolation or capture is contemplated, the capture module is immobilized or has means for immobilization. Whilst modulating modules may

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be immobilized or carry means for immobilization, it will be appreciated that these will then effectively function as the capture module.

The means for immobilization may be inherently part of the nucleic acid sequence of the capture module, for example a poly T tail may be provided to bind to a solid support carrying a complementary oligo dA sequence. It will be appreciated that it is inadvisable to use a capture module with a poly A tail to be bound to a support carrying an oligo dT sequence as to do so may lead to the capture of mRNA which may be present in the sample. Other specific sequences which are complementary to sequences which can be attached directly or indirectly to an immobilizing support may also form part of the capture module for the purposes of immobilization.

The above methods involve the addition of further nucleotides to the capture module over those required for binding to the target nucleic acid. Extensions in this way are not always necessary and the means for immobilization may be introduced during or post oligonucleotide synthesis to nucleotides of the capture module to allow direct or indirect attachment to an immobilizing support through a binding partner. Conveniently, derivatized nucleotides may be used during synthesis to provide the appropriate first partner of the binding pair. The second partner of the binding pair is then carried on the support. Suitably derivatized capture oligonucleotides thus include those carrying biotin for binding to avidin or streptavidin, carrying epitopes or haptens (eg. digoxigenin) for binding to antibodies (which may be mono- or polyclonal) or antibody fragments or carrying DNA sequences for binding to DNA or PNA binding proteins (eg. the lac I repressor protein binding to a lac operator sequence attached to the oligonucleotide). Other suitable pairings include protein A-antibody, protein G-human serum albumin (HSA) and functional parts thereof. It will be appreciated

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that either of the partners of the binding pairs noted above, or functional parts thereof, may bind to the oligonucleotide. The streptavidin/biotin binding system is very commonly used in molecular biology, due to the relative ease with which biotin can be incorporated within nucleotide sequences, and indeed the commercial availability of biotin-labelled nucleotides, and thus this represents one preferred method for attachment of the capture module to the support.

Numerous suitable supports for immobilization of oligonucleotides, and methods of attaching nucleotides to them, are well known in the art and widely described in the literature. Thus for example, supports in the form of sheets, gels, filters, membranes, microfibre strips, plates, microtitre wells, tubes, dipsticks, particles, fibres or capillaries may be used, made of a polymeric material for example of agarose, cellulose, alginate, teflon[™], latex or polystyrene. Particulate materials, especially beads, are generally preferred. For example, sepharose[™] or polystyrene beads may be used. Advantageously, the support may comprise magnetic particles, eg. the superparamagnetic beads produced by Dynal AS (Oslo, Norway) and sold under the trademark DYNABEADS[™]. Chips may be used as solid supports to provide miniature experimental systems as described for example in Nilsson et al. (1995, Anal. Biochem., 224, p400-408).

The solid support may carry functional groups such as hydroxyl, carboxyl, aldehyde or amino groups for the attachment of the capture module. These may in general be provided by treating the support to provide a surface coating of a polymer carrying one of such functional groups, eg. polyurethane together with a polyglycol to provide hydroxyl groups, or a cellulose derivative to provide hydroxyl groups, a polymer or copolymer of acrylic acid or methacrylic acid to provide carboxyl groups or an amino alkylated polymer to provide amino

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groups. US patent No. 4,654,267 describes the introduction of many such surface coatings.

Alternatively, the support may carry other moieties for attachment, such as avidin or streptavidin, DNA binding proteins or antibodies or antibody fragments. Streptavidin-coated DYNABEADS are commercially available from Dynal AS. Preferably, immobilizing oligonucleotides are produced which bear a biotin moiety which may be used to attach to streptavidin on a solid support.

When detection of target nucleic acid is contemplated, which may or may not follow an isolation or capture method according to the invention, at least one of the modules of the modular oligonucleotide may be labelled.

The term "label" as used herein refers to any label which can be assessed qualitatively or quantitatively, directly or indirectly, eg. by virtue of its enzymatic properties, radiation emission, scattering or absorption properties, or of its ability to cooperate with or bind to a complimentary agent to produce a detectable effect, eg. interact with an enzyme to produce a signal, gas evolution, light emission, colour change, turbidity, precipitations etc. Such labels or means for labelling are well known, especially in the field of diagnostic assays and include for example, enzymes, chromophores or fluorophores (eg. dyes such as fluorescein and rhodamine), radiolabels, chemiluminescent compounds or reagents of high electron density such as ferritin, haemocyanin or colloidal gold. A label which uses enzyme activity to generate a colour for spectrophotometric assessment may be employed, for example β -galactosidase, alkaline phosphatase or peroxidase which on the addition of a suitable substrate may generate a signal suitable for detection.

Labels are conveniently introduced into parts of the modular oligonucleotide during or post synthesis. This may be achieved in a similar manner to providing a

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means for immobilization, by for example providing the oligonucleotide with one partner of a binding pair (pre or post synthesis), and subsequently attaching a second binding partner provided with a label. The first partner may be one of a conventional binding pair, for example biotin:streptavidin or may be part of the oligonucleotide sequence itself to which a second molecule will bind specifically. Alternatively, a derivatized nucleotide bearing a label, for example a radiolabelled nucleotide, may be used in the synthesis of the oligonucleotide or derivatized after synthesis. Alternatively, a module may be synthesized with a portion which is not complementary to the target nucleic acid which may inherently carry a label, e.g. a radiolabel, or be suitable for the attachment of a label. Such extensions to a module are not considered as part of the module when determining if improved binding is observed for a modular oligonucleotide compared to a single oligonucleotide spanning these modules, according to the definition of the invention.

Whilst detection may be achieved by using one or more labelled modules of the modular oligonucleotide to indicate binding, such a method has the disadvantage that the labelled modules bound to target nucleic acid must necessarily be separated from the binding reaction mix for detection above background levels to be possible. Although this separation may in most cases be performed readily, an alternative method of detection involves the use of labels on modules which bind to adjacent stretches of the target, which by their proximity generate a signal (negatively or positively) which may be detected. Such a label has the advantage not only that separation need not be performed for detection, although this may additionally be performed if required, but also that the signal is created only when modules bind adjacent to one another, thus reducing background noise. For example, modules with different labels may be used in which the

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labels are of sufficient proximity and suitable type that when the modules are bound to the target nucleic acid, they quench the possible fluorescence of the other label. Thus for example, two modules with different labels may be used, one of which is a quencher dye and the other a fluorescent dye. When not bound adjacent to one another fluorescence will occur, whereas when bound adjacent a measurable decrease in fluorescence may occur.

Optionally, the target nucleic acid with bound quenching modules may be separated from unbound modules in the mixture. The bound molecules may then be released e.g. by heating to disrupt binding, thereby causing fluorescence as the labels on the modules separate allowing detectable fluorescence which may be correlated to the amount of the module bound and hence the amount of target DNA. Such labels are used in the TaqMan assay (Perkin Elmer).

It will however be appreciated that detection does not always rely on labelling the modules. For example, chip technology may be used, as described herein, in which the capture module of the modular probe is attached to the surface of the chip (see for example Nilsson et al., 1995, supra). When the capture module binds to the target DNA (in the presence of the modulating modules) a change in refractive index occurs at the sensor surface. This change correlates to the amount of target bound to the chip and thus may be used as a method of detection and/or isolation or capture. This method represents a preferred feature of the invention.

For performance of the invention, the method may additionally include the further step of attaching a capture module to a solid support in instances in which the module is provided with means for immobilization, prior or subsequent to the binding of the capture oligonucleotide to target nucleic acid by contacting the sample containing the target molecule with the immobilized capture oligonucleotide. Once bound to a

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solid support, washing steps may conveniently be performed, especially for purification purposes or to remove background in detection steps. Preferably, the capture oligonucleotide is bound to a solid support prior to the addition of a sample containing target nucleic acid molecules.

In methods of the invention, modulating modules are preferably added to, or contacted with, a sample containing the target nucleic acid molecules prior to the addition of the free or immobilized capture module, for example by mixing together at 54°C for 45 minutes followed by cooling to room temperature to allow hybridization.

In procedures employing methods of the invention, especially assay procedures, additional steps of isolation, separation, purification, assessment and/or comparison may be performed as appropriate to obtain the desired results. Thus, for example a method of the invention may comprise at least one of the following additional steps:

- a) attaching the capture module of the modular oligonucleotide to a solid support in instances in which the capture module is provided with a means for immobilization;
- b) contacting the sample containing the target nucleic acid with the modular oligonucleotide;
- c) contacting the sample containing the target nucleic acid with the modulating modules of the modular oligonucleotide;
- d) contacting the sample containing the target nucleic acid with the immobilized capture oligonucleotide to allow binding of the oligonucleotide to target nucleic acid;
- e) separating target nucleic acid bound to the capture module from the sample;
- f) washing the target nucleic acid separated in (e) above;

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- g) assessing the presence or amount of label associated with the target nucleic acid, when labelled modules are used, or assessing the presence or amount of target nucleic acid bound to the capture module when no label is employed; and
- h) comparing the amount of label, or bound target nucleic acid of (g) with control levels.

It will be appreciated that not all of the above steps may be incorporated into any given procedure as for example, steps (c) and (d) essentially perform step (b) in two parts. In step (g), assessment of label or bound target molecules may alternatively be performed by assessment of label not associated with target molecules or assessment of unbound nucleic acid, which values may then be subtracted from total values of label or nucleic acid used to give the required value of label or bound target nucleic acid. Whilst these values may be correlated to appropriate standard curves to obtain absolute values, this is not essential, and the term "assessing" as used herein includes both quantitation in the sense of obtaining an absolute value for the amount of target nucleic acid in a sample, and also obtaining a semi-quantitative or qualitative assessment, for example to indicate simply the presence of target nucleic acid in the sample under study. Assessment may also involve the generation of further molecules for detection, for example by sequencing and/or amplification reactions. With regard to step (h), suitable control levels will be those established using the same experimental procedures for non-test or normal samples. It will be clear that different sequential steps may be employed to achieve binding of the modular oligonucleotide. For example, a part of the modular oligonucleotide may be contacted with the sample and then bound, followed by contacting and binding of further parts of the modular oligonucleotide. Alternatively the contacting steps may be performed simultaneously.

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In a preferred aspect of the invention, the method may comprise the steps of:

- 1) contacting the sample containing the target nucleic acid with all modules of the modular oligonucleotide;
- 2) binding said modules by hybridization;
- 3) addition of a solid support and attachment of at least one of said modules provided with a means for immobilization to said solid support;
- 4) separating target nucleic acid bound to said solid support;
- 5) washing said solid support;
- 6) amplification of said target nucleic acid; and
- 7) assessing the presence or amount of amplified nucleic acid.

It will be appreciated that modular oligonucleotides which have utility according to the invention may be made up of different numbers of modules (with or without spaces between them when bound to target nucleic acid molecules), each of which may be different sizes. Whilst this invention has been found to have utility when tested at different target DNA sites, some appropriate modification of module number and/or module size may be appropriate to obtain optimum binding at a given site under particular experimental conditions. Such optimization is within the scope of the skilled addressee in which trial and error experiments of the type illustrated herein may be employed. Thus, in general, 5 or fewer modules make up the modular oligonucleotide, preferably 2 or 3 modules, with each module containing 4 or more nucleotide bases. Preferably the modular oligonucleotide contains a total of at least 10 nucleotides, preferably at least 18, for example 18, 24, 27, 29, 31, 33 or 36. Modules, when bound to target nucleic acid are preferably less than 10 nucleotide bases apart, especially preferably less than 2 bases apart, particularly preferably, without any bases in between.

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It is especially preferred that less than 2 bases separate the capture module and first adjacent modulating module when bound to the target.

Specifically preferred features of modular oligonucleotides for use in the invention are those with 2 or 3 modules, each module with > 5 nucleotides, preferably $\geq 9 \leq 18$, eg. 9, 11, 13, 15 or 18 nucleotides. When 3 modules are employed, slightly shorter modules may be used than when 2 modules are used such that the total nucleotides in 2-part modular oligonucleotides are > 27 , preferably ≥ 29 , eg. 29, 31, 33 or 36 and in 3-part modular oligonucleotides > 23 , preferably ≥ 27 , eg. 27, 31, 33 or 36 nucleotides.

The modules of the modular oligonucleotides may be prepared by chemical or other appropriate synthesis well known in the art. Several useful oligonucleotides are available commercially with an attached biotin molecule for immobilization (e.g. KEBO, Stockholm, Sweden).

The method has utility especially with regard to viral target nucleic acid, for example Hepatitis C virus (HCV) and may be used to monitor or diagnose viral or other infections. Suitable modular oligonucleotides for use in methods of the invention include modular oligonucleotides having one of the following sequences: For detection, isolation or capture of HCV at positions 291-341:-

H1-18 + C1 (18+18)

3'-ACGCTCACGGGGCCCTCC-5' + 3'-AGAGCATCTGGCACGTGG-5'

H2 + C1 (11+18)

3'-ACGGGGCCCTC-5' + 3'-AGAGCATCTGGCACGTGG-5'

H1-15 + C1 (15+18)

3'-CTCACGGGGCCCTCC-5' + 3'-AGAGCATCTGGCACGTGG-5'

H1-13 + C1 (13+18)

3'-CACGGGGCCCTCC-5' + 3'-AGAGCATCTGGCACGTGG-5'

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H1-11 + C1 (11+18)

3'-CGGGGCCCTCC-5' + 3'-AGAGCATCTGGCACGTGG-5'

H8 + H1-9 + C1 (9+9+18)

3'-ACGCTCACG-5' + 3'-GGGCCCTCC-5' +

3'-AGAGCATCTGGCACGTGG-5'

H1-18 + H4 + C2 (18+9+9)

3'-ACGCTCACGGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'

H1-15 + H4 + C2 (15+9+9)

3'-CTCACGGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'

H1-13 + H4 + C2 (13+9+9)

3'-CACGGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'

H1-11 + H4 + C2 (11+9+9)

3'-CGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'

H1-9 + H4 + C2 (9+9+9)

3'-GGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'

H3 + H5 + C2 (9+9+9)

3'-GGGGCCCTC-5' + 3'-CAGAGCATC-5' + 3'-GGCACGTGG-5'

H3 + H4 + C2 (9+9+9)

3'-GGGGCCCTC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'

For detection, isolation or capture of HCV at positions 132-167:-

OMD6 + OMD2 (18+18)

3'-CCTCTCGGTATCACCAGA-5' + 3'-CGCCTTGGCCACTCATGT-5'

Preferably in the above modular oligonucleotides, the last listed module is the capture module (ie. C1, C2

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or OMD2) and may bear a moiety for immobilization, preferably a biotin molecule at the 5' end. These modular oligonucleotides and others suitable for use in methods of the invention form further aspects of the invention. Thus in a yet still further aspect, the present invention provides methods of the invention for detecting and/or isolating HCV, wherein said oligonucleotides comprise one of the following nucleotide sequences:

3'-ACGCTCACGGGGCCCTCC-5' + 3'-AGAGCATCTGGCACGTGG-5'; or
 3'-ACGGGGCCCTC-5' + 3'-AGAGCATCTGGCACGTGG-5'; or
 3'-CTCACGGGGCCCTCC-5' + 3'-AGAGCATCTGGCACGTGG-5'; or
 3'-CACGGGGCCCTCC-5' + 3'-AGAGCATCTGGCACGTGG-5'; or
 3'-CGGGGCCCTCC-5' + 3'-AGAGCATCTGGCACGTGG-5'; or
 3'-ACGCTCACG-5' + 3'-GGGCCCTCC-5' +
 3'-AGAGCATCTGGCACGTGG-5'; or
 3'-ACGCTCACGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-
 GGCACGTGG-5'; or
 3'-CTCACGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-
 5'; or
 3'-CACGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5';
 or
 3'-CGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'; or
 3'-GGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'; or
 3'-GGGGCCCTC-5' + 3'-CAGAGCATC-5' + 3'-GGCACGTGG-5'; or
 3'-GGGGCCCTC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5'; or
 3'-CCTCTCGGTATCACCAGA-5' + 3'-CGCCTTGGCCACTCATGT-5',
 or analogs or derivatives thereof.

The invention also has utility with regard to identifying and/or isolating target HIV nucleic acid. In this respect, modular probes directed to the polymerase region of HIV-1 have been designed to allow capture and/or isolation of the HIV RNA genome for diagnostic purposes. Although HIV RNA contains poly A which enables purification by binding to a solid support bearing oligo-dT, conveniently using the method of the invention, a

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capture oligonucleotide is used which binds adjacent to the site where RT-PCR primers would be located such that the effects of RNases are minimized. In this respect, suitable modular oligonucleotides for use in the methods of the invention include modular oligonucleotides having one of the following sequences:

OMD82x13 + OMD83 (13+18)

3'-TTAATTTTCGGTCC-5' + 3'-TTACCTACCGGGTTTCA-5'

OMD81 + OMD82 (18+18)

3'-AGGATAACTTTGACATGG-5' + 3'-TCATTTTAATTTTCGGTCC-5'.

Preferably in the above modular oligonucleotides, the last listed module is the capture module (ie. OMD82, 83) and may bear a moiety for immobilization, preferably a biotin molecule at the 5' end. These modular oligonucleotides and others suitable for use in methods of the invention form further aspects of the invention. Thus in a yet still further aspect, the present invention provides methods of the invention for detecting and/or isolating HIV, wherein said oligonucleotides comprise one of the following nucleotide sequences:

3'-TTAATTTTCGGTCC-5' + 3'-TTACCTACCGGGTTTCA-5'; or
3'-AGGATAACTTTGACATGG-5' + 3'-TCATTTTAATTTTCGGTCC-5',
or analogs or derivatives thereof.

The invention also has utility with regard to identifying and/or isolating sequencing products generated by the extension of primers, for example the universal sequencing primer (USP). Such products need to be purified and/or enriched after synthesis before loading onto an electrophoresis system. Although alcohol precipitation is routinely used, this is not easy to automate. The use of modular probes allows the capture of such products as generated by, for example,

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traditional T7 DNA polymerase sequencing or cycle sequencing protocols. It will be appreciated that this may be adapted for use with respect to different primers used to synthesize extension products. Thus, for capture of the products of universal primer extension reactions, suitable modular oligonucleotides for use in methods of the invention include modular oligonucleotides having one of the following sequences:

JL-H1/USP + JL-C2/USP (9+9)

3'-GACGTCCAG-5' + 3'-CTGAGATCT-5'

JL-H2/USP + JL-C1/USP (13+18)

3'-GTTTCGAACGTACG-5' + 3'-GACGTCCAGCTGAGATCT-5'

JL-H2/USP + JL-H1/USP + JL-C2/USP (13+9+9)

3'-GTTTCGAACGTACG-5' + 3'-GACGTCCAG-5' + 3'-CTGAGATCT-5'

Preferably in the above modular oligonucleotides, the last listed module is the capture module (ie. JL-C1/USP, JL-C2/USP) and may bear a moiety for immobilization, preferably a biotin molecule at the 5' end. These modular oligonucleotides and others suitable for use in methods of the invention form further aspects of the invention. Thus in a yet still further aspect, the present invention provides methods of the invention for detecting and/or isolating primer extension products, especially of the primer, USP, wherein said oligonucleotides comprise one of the following nucleotide sequences:

3'-GACGTCCAG-5' + 3'-CTGAGATCT-5'; or

3'-GTTTCGAACGTACG-5' + 3'-GACGTCCAGCTGAGATCT-5'; or

3'-GTTTCGAACGTACG-5' + 3'-GACGTCCAG-5' + 3'-CTGAGATCT-5',

or analogs or derivatives thereof.

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Such analogs and derivatives include modified or derivatized nucleotide bases or oligonucleotides as referred to previously, which retain their ability to fulfill the complementarity requirements described herein and which include for example, nucleotides bearing labels or means for immobilization. Alternatively, particular bases defined above may be replaced by other non-complementary bases or derivatized bases which do not prevent binding to the target nucleic acid to such an extent as to fall outside the definition of complementarity as described herein.

In a further aspect, the invention also provides the modular oligonucleotides as described herein and their use in methods of the invention.

The present invention also extends to kits for performing the methods of the invention, comprising at least the following:-

modular oligonucleotides having two or more parts, suitable for use in methods of the invention.

Preferably, at least one of the modules is immobilized on a solid support or has means for immobilization. At least one of the modules may be labelled to allow detection of the target nucleic acid. Additionally, appropriate buffers and/or a solid support may be provided.

The following Examples are given by way of illustration only with reference to the following Figures in which:-

Figure 1 shows a typical sensorgram;

Figure 2A shows a schematic representation of viral capture with an oligonucleotide module (= hybridising probe) (18-5 mer) injected over an immobilised 18-mer capture oligonucleotide (= immobilised probe) on the chip surface;

Figure 2B shows the results of capture using the 18-mer capture oligonucleotide (* denotes capture data when an 18 nucleotide gap was present between the capture

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oligonucleotide and the oligonucleotide module - column 11);

Figure 3A shows a schematic representation of viral capture with 2 oligonucleotide modules of the modular probe (9 mer and 18-5 mer) injected over an immobilised 9 mer capture oligonucleotide (* denotes capture data in the absence of the oligonucleotide module H4 - column 1; column 12 presents capture data when an 18 nucleotide gap was present between the oligonucleotide modules);

Figure 3B shows the results of capture using the 9-mer capture oligonucleotide;

Figure 4A is as in Figure 3A except for the presence of a 1 nucleotide space between the capture oligonucleotide and the first adjacent oligonucleotide of the modular probe or between the two non-capture oligonucleotide modules of the modular probe;

Figure 4B shows the results of capture using a modular probe with gaps between the modules;

Figures 5A and B show the effect of different numbers of modules comprising the modular probe;

Figure 6 shows the modular effect in location 2 of the HCV genome;

Figure 7 shows a schematic representation of the use of modular oligonucleotides to capture HCV DNA or RNA;

Figure 8 shows the results of capture of HCV DNA onto magnetic beads in the absence or presence of an oligonucleotide module;

Figure 9 shows BIAcore analysis results of capture of HCV RNA onto the chip surface in the absence or presence of an oligonucleotide module;

Figure 10 shows the results of capture of HCV RNA onto magnetic beads in the absence or presence of an oligonucleotide module after single PCR;

Figure 11 shows the results of capture of HCV RNA onto magnetic beads in the absence or presence of an oligonucleotide module after nested PCR;

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Figure 12 shows the results of capture of HCV RNA from clinical hepatitis C samples onto magnetic beads in the absence or presence of an oligonucleotide module after single PCR; and

Figure 13 shows BIAcore analysis results of capture of ss HIV-1 DNA in the absence or presence of an oligonucleotide module onto the chip surface.

EXAMPLE 1: Capture of ssDNA using a capture oligonucleotide and at least one additional oligonucleotide as the modular probe.

This example illustrates the manyfold increase in the capture of ssDNA by an immobilised capture oligonucleotide when specific oligonucleotide modules have been previously hybridised to the DNA. The capture oligonucleotide (referred to in this example as capture oligonucleotide or immobilized oligonucleotide) and oligonucleotide hybridized to the DNA (referred to in this example as oligonucleotide modules) together are the modules of the modular probe.

Various combinations of capturing oligonucleotides and oligonucleotide modules for binding to the DNA were used. Thus, 18 mer capture oligonucleotides were used in conjunction with either an 18, 15, 13, 11, 9 or 5 mer oligonucleotide module (Figure 2B), 9 mer capturing oligonucleotides with a 9 mer oligonucleotide module with and without a second oligonucleotide module (18, 15, 13, 11, 9 or 5 mer) (Figure 3B). Also modular probes with a 1 nucleotide space between the annealing sites of the modules were tested (Figure 4B and 5A). To further investigate the modular effect a 36 mer biotinylated oligonucleotide (comprising of the 18 mer capture oligonucleotide C1 and the 18 mer oligonucleotide module H1-18) was designed and tested for its efficiency in capturing HCV (Figure 5B).

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MATERIALS AND METHODS*PCR amplification (Template generation)*

Clones containing the 5' non-translated region (NTR) of two hepatitis C virus (HCV) genotypes (2b and 3a) in the pGEM®-T vectors (Promega, Madison, WI, USA), were used as a template in the PCR to generate a 324 bp fragment for biosensor analysis. PCR amplification was performed with 0.2 μ M of each primer OU49 and OD66;

OU49 5'-GGCGACACTCCACCATGAATC-3'

OD66 5'-biotin-GGTGCACGGTCTACGAGACC-3'

Amplification was performed in a 50 μ l reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.1 % Tween® 20, 0.2 mM dNTP's and 0.5 U of AmpliTaq® DNA polymerase (Perkin-Elmer, Foster City, CA), using a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Norwalk, CT). The temperature profile was 94°C for 5 minutes, followed by 30 cycles of (94°C for 15 seconds, 62°C for 45 seconds, 72°C for 60 seconds) and ending with 72°C for 10 minutes.

Single strand preparation

The biotinylated PCR-products were immobilised onto streptavidin-coated paramagnetic beads (Dynabeads® M-280 Streptavidin; Dynal, Oslo, Norway) and by strand-specific elution a pure template for hybridisation was obtained (Hultman et al., 1989, Nucl. Acids Res., 17, p 4937-4946). Fifty microlitres of PCR-product was captured by incubation for 15 minutes at room temperature with 5 mg/ml of beads in 50 μ l binding/washing buffer (10 mM Tris-HCl (pH 7.5) 1 mM EDTA, 2 M NaCl, 1 mM β -mercaptoethanol, 0.1 % Tween® 20). After washing and removal of supernatant, the strands were separated by incubation with 10 μ l of 0.1 M NaOH for 5 minutes. The alkaline supernatant with the non-biotinylated strand was neutralised with 6 μ l of 0.1667 mM HCl and 1 μ l of 280 mM

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Tris-HCl, pH 7.5, 100 mM MgCl₂. In order to prevent any co-eluted biotinylated strands from interacting with the streptavidin on the sensor chip, a second round of sedimented streptavidin beads (250 µg) was mixed and the supernatant collected. In order to reduce the differences between individual samples within an experiment the eluted single strand DNA was batched. The prepared single stranded DNA was then qualitatively and quantitatively analysed on 10-15% PAGE with PhastSystemTM and PhastGel[®] DNA Buffer Strips and DNA Silver Staining Kit (Pharmacia Biotech, Uppsala, Sweden).

Oligonucleotides

Biotinylated oligonucleotides for immobilisation onto the sensor chip and oligonucleotides for hybridisation to the ss HCV PCR product were purchased from KEBO, Stockholm, Sweden. The oligonucleotide sequences are shown in Table 1 for location 1 and Table 2 for location 2.

Biosensor analysis

A BIAcore[®] 2000 instrument (Pharmacia Biosensor, Uppsala, Sweden) was used in all experiments. Sensor chips SA (Pharmacia Biosensor), precoated with approximately 4000 RU streptavidin (1000 RU corresponds to approximately 1 ng/mm² of streptavidin), were used. Experiments were performed at 25°C with 6X SSPE (0.9M NaCl (pH 7.4), 60 mM NaH₂PO₄, 7.5 mM EDTA and 0.005% (v/v) Surfactant P20 (Pharmacia Biosensor)) as injection and running buffer. The biotinylated oligonucleotides for capture onto the sensor chip were immobilised to a level of approximately 500 - 1000 RU (1000 RU corresponds to approximately 1 ng/mm² (Stenberg et al., 1991, J. Colloid Interface Sci., 143, p 513-526) by injection of 50 µl 6X SSPE containing 1 µM biotinylated oligonucleotide at a flow of 30 µl/minute. Before and after use in hybridisation experiments the sensor chips were treated with three pulses of 50 mM NaOH (5 µl, 5µl/min) to regenerate (R)

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the surface. One flow cell, without immobilized oligonucleotide was used as reference.

Hybridisation and single strand capture

Oligonucleotide modules were hybridised to the ss target DNA by incubation in a hybridisation oven for 45 minutes at 54°C with constant rotation then cooled to room temperature. Hybridisation was performed in 100 μ l 6x SSPE containing approximately 200 nM ssDNA and 500 nM oligonucleotides.

Forty μ l of the hybridisation mix was injected over the immobilised capture oligonucleotide at a flow of 5 μ l/min. Controls samples with no hybridizing probes were treated in exactly the same manner. The oligonucleotide surface was regenerated with 50 mM NaOH (5 μ l, 5 μ l/min).

RESULTS

A 324 base pair (bp) fragment of the non-translated region (NTR) of HCV was generated by PCR. The dsDNA was melted apart by magnetic bead separation to obtain ssDNA suitable for capture by oligonucleotides immobilised on a sensor chip in the biosensor. This ssDNA was hybridised to oligonucleotide modules prior to injection over the sensor chip which results in a highly efficient capture of the HCV DNA.

In this example biosensor technology was used which allows biological events to be monitored in real time (Jönsson et al., 1991, BioTechniques, 11, 620-672). This method utilises a sensor chip as a solid support for immobilisation. Detection is based on surface plasmon resonance (SPR) to monitor changes in refractive index over time at the sensor surface. The changes are proportional to the mass of molecules bound on the surface and are shown in a so-called sensorgram as resonance units (RU) over time. A representative

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sensogram is depicted in Figure 1 which shows the injection (A) of the biotinylated C1 (18 mer capture oligonucleotide). The immobilisation is rapid and the amount of bound oligonucleotide is determined to be 700 RU by comparing the response units before and after the injection pulse. After regeneration (R) of the sensor surface with 50 mM NaOH, the immobilised capture oligonucleotide was used for capturing the single strand DNA target by hybridisation. As shown in Figure 1 (B) when only single strand target DNA is injected over the sensor chip only negligible amounts are hybridised (<20 RU). In contrast, when the single strand target with a preannealed oligonucleotide module H1 (18 mer), designed to be adjacent to the capture oligonucleotide, was passed over the sensor chip (C), significant amounts were retained (250 RU).

Investigation of the modular effect using an 18 mer oligonucleotide as capture probe in location 1.

An 18 mer biotinylated capture oligonucleotide (C1) was immobilised on a Streptavidin sensor chip. Single stranded HCV DNA with and without previously hybridised oligonucleotide modules was injected over the sensor chip as described in the methods section. The experimental protocol is illustrated schematically in Figure 2A and the results are shown in Figure 2B. The results represent normalised values from independent experiments as a consequence of the variation of absolute responses between different sensor chips, depending on the amount of streptavidin coated onto the chip surface, thus affecting the amount of immobilised capture oligonucleotide and capture efficiency. The low capture efficiency with ss DNA injected over an 18 mer immobilised capture oligonucleotide (C1) is displayed in Figure 2B, column 1. A similar low capture was also achieved even if a 5' spacer of 10 adenines was used with the 18 mer (C1x10A, Table 1) (data not shown). In the

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subsequent capture experiments a prehybridised complex comprising of ss target DNA and oligonucleotide modules was used. The oligonucleotide modules varied from 18 to 5 nucleotides in length, but all were designed to anneal adjacent to the 3'-end of the immobilised capture oligonucleotide (Table 1). Using the specific oligonucleotide modules (H1-18, 15, 13, 11; 18 to 11 mers) a significant increase of capture (230 to 320 RUs) was observed (Figure 2B, columns 2-5), as compared with the experiment lacking an oligonucleotide module (column 1). However, the capture efficiency was greatly diminished and sometimes abolished when H1 was less than 9 nucleotides in length (Figure 2B, column 6 and 7). The different controls clearly indicate the specificity on hybridisation between the immobilised capture oligonucleotide and the single strand DNA/oligonucleotide module complex as the responses arising from interactions of oligonucleotides or nonspecific DNA were negligible (Figure 2B, columns 8 to 11). Column 11 shows that no capture occurs when an 18 nucleotide gap is present between the different modules.

Investigation of the modular effect using a 9 mer oligonucleotide as capture probe.

A 9 mer biotinylated capture oligonucleotide (C2) was immobilised on a SA sensor chip and the oligonucleotide modules fractionated into smaller modules. The experimental protocol is illustrated schematically in Figure 3A and the results are shown in Figure 3B. As expected no capture of DNA was observed when an immobilised capture nonamer (C2) was used alone (Figure 3B, column 1). When the nonamer oligonucleotide module (H4) and the oligonucleotide module (H1 - 18, 15, 13, 11, 9) were used, ss DNA was successfully captured (Figure 3B, columns 3-7). However as in Figure 2B, module assisted capture with the short pentamer module (H1-5) was unsuccessful (column 8) as well as use of a single

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nonamer oligonucleotide module (H4) (column 2). These results together with data from Figures 5A and 5B, suggest that it is not the length of the capture oligonucleotide that is the most important parameter, rather it depends on the number and length of oligonucleotide modules that are employed. Control experiments were carried out both for the 18 and 9 mer immobilised capture oligonucleotides to verify the concept. Firstly, non-specific interactions were investigated by injecting a similar sized non-specific DNA over the capture oligonucleotide (Figure 2B, column 8, and Figure 3B, column 10) and by injecting the oligonucleotide module alone (Figure 2B, column 9 and Figure 3B, column 11). No increase in response was observed. Secondly, non-specific DNA was co-incubated with the target DNA and the oligonucleotide module. Capture was still specific and no interference from the non-specific DNA was observed (Figure 2B, column 10; Figure 3B, column 9). Hence the modular approach has the ability to capture a specific target without any reduction in signal when challenged with unrelated DNA. Column 12 indicates that no capture occurs when an 18 nucleotide gap is present between the non-capture oligonucleotide modules.

The effect of gaps between oligonucleotide modules

From the previous experiments the detrimental effects on capture efficiency with 18 nucleotide long gaps between oligonucleotide modules was clear (Figure 3B, column 12) and/or immobilised capture probe (Figure 2B, column 11). To further analyse the restrictions in the oligonucleotide module assisted capture approach, single nucleotide gaps were introduced between the modules of the modular probe. The H1-11 mer capture oligonucleotide and the two nonamer oligonucleotide modules (H4 and H1-9) were reconstructed and their annealing sites shifted one nucleotide towards the 5' end of the target DNA and were

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renamed H2, H5 and H3 respectively (Table 1). Comparison showed that discontinuous probes were able to capture single strand target, although at a lower efficiency (Figure 4A, B).

Fragmentation of long oligonucleotides into shorter modules

This experiment was performed to further investigate whether the efficiency could be improved by fragmentation of extended capture oligonucleotides into shorter modular units. As illustrated in Figure 5A (columns 1, 2 and 3) when an immobilized 27 mer oligonucleotide was used for capturing or when two oligonucleotides with a total annealing length of 27 nucleotides were used, the capture of DNA was poor. However when this 27 nucleotide stretch was fragmented into three nonamers a highly efficient capture was observed (Figure 5A, column 4). This was further substantiated by a 36 nucleotide capture oligonucleotide (C4) (Table 1) which fails to efficiently capture single strand target DNA (Figure 5B, column 1), while use of oligonucleotide modules and a shorter immobilised capture probe significantly improves the capture (Figure 5B, columns 2, 3 and 4).

To investigate whether this effect is observed at another position in the HCV genome (location 2), a second 18 mer capturing oligonucleotide was designed together with an 18 mer oligonucleotide module. The oligonucleotide sequences are shown in Table 2.

Investigation of the modular effect in location 2 of the HCV genome.

An 18 mer biotinylated capture oligonucleotide (OMD2) was immobilised on a SA sensor chip as described above. The results are shown in Figure 6. Injection of ssDNA with an 18 mer oligonucleotide module (OMD6) resulted in an increase of 400 RU (column 2) while ssHCV alone was not

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captured (column 1).

CONCLUSIONS

This example illustrates that ssHCV is captured poorly or not at all by the 18 mer oligonucleotide immobilised on the chip (Figure 2B, column 1, Figure 4B, column 3 and Figure 5B, column 5). When the ssHCV was incubated with an oligonucleotide module of 18, 15, 13 or 11 nucleotides a significant increase in capture was observed (Figure 2B, columns 2-5). However incubation of ssHCV DNA with a 9 mer or 5 mer oligonucleotide module did not result in an increase in capture (Figure 2B, columns 6-7). When a 36 mer capture oligonucleotide was used in place of the 18 mer capture oligonucleotide and the 18 mer oligonucleotide module little capture of HCV was observed (Figure 5B, column 1). These results further substantiate the modular effect. Non-specific hybridisation of the HCV DNA was investigated by injecting a similar sized non-HCV ss PCR product (treated in the same manner) over the capture oligonucleotide (Figure 2B, column 8). Non-specific hybridisation of the oligonucleotide modules was investigated and was also ruled out (Figure 2B, column 9).

No capture of DNA was observed when a 9 mer immobilised capture oligonucleotide was used alone for capture (Figure 3B, column 1) or with a single 9 mer oligonucleotide module (Figure 3B, column 2). However, when two 9 mer oligonucleotide modules were incubated with the DNA, a highly efficient capture was observed by the immobilised 9 mer (Figure 3B, column 7). These oligonucleotides anneal at the same position on the DNA as the 18 mer capturing oligonucleotide with the 9 mer oligonucleotide module (Figure 2B, column 6) but hybridisation is only observed when 2 oligonucleotide modules are used as opposed to 1 oligonucleotide module.

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When an 18 and a 9 nucleotide space between the second and third oligonucleotides of the modular probes was inserted the modular effect was abolished (Figure 3B, column 12, data for the 9-nucleotide gap not shown). To further investigate the effect of inserting a space between the modules, three 9 mer oligonucleotides with a one nucleotide gap between them were designed. These one nucleotide gaps do seem to result in slightly less capture of HCV DNA but nevertheless good hybridisation signals of approximately 50 to 100 RU were obtained. (Figure 4B, columns 5 and 6). The gap between the capturing oligonucleotide and the first oligonucleotide module (column 5) resulted in slightly more efficient capture than the gap between the second and third modules (column 6). A single gap between the modules of a modular probe with 2 modules also resulted in improved capture over the non-modular probe, but was reduced with respect to a 2-module modular probe without a gap between modules (Figure 4B, columns 1 and 2).

The results for experiments using modular probes complementary to location 2 further support the modular theory, as a 200 fold increase in signal was observed when the 18 mer oligonucleotide module was hybridised to the DNA prior to capture by the immobilised oligonucleotide (Figure 6).

Table 1 - Oligonucleotides used in the module assisted capture of target DNA - location 1

HCV Seq	5'-	290	300	310	320	330	340	-3'
		---	TGCCTGATAGGTGCTTGCGAGTGCCCGGAGGTCTCGTAGACCGTGCACC---					
C1	3'-							-biotin-5'
C1x10A	3'-					AGAGCATCTGGCACGTGG		-biotin-5'
C2	3'-					AGAGCATCTGGCACGTGG(A) ₁₀		-biotin-5'
C3	3'-					GGCACGTGG		-biotin-5'
C4	3'-				GGGCCCTCCAGAGCATCTGGCACGTGG			-biotin-5'
				ACGCTACGGGGCCCTCCAGAGCATCTGGCACGTGG				-biotin-5'
H1-18	3'-			ACGCTACGGGGCCCTCC				-5'
H1-15	3'-			CTCACGGGGCCCTCC				-5'
H1-13	3'-			CACGGGGCCCTCC				-5'
H1-11	3'-			CGGGGGCCCTCC				-5'
H1-9	3'-			GGGGCCCTCC				-5'
H1-5	3'-			CCTCC				-5'
H2	3'-			ACGGGGCCCTC				-5'
H3	3'-			GGGGCCCTC				-5'
H4	3'-					AGAGCATCT		-5'
H5	3'-					CAGAGCATC		-5'
H6	3'-					GGGCCCTCCAGAGCATCT		-5'
H7	3'-	CGGACTATCCACGA						-5'
H8	3'-		ACGCTCAG					-5'

Table 2 - Oligonucleotides used in the module assisted capture of target DNA - location 2

HCV seq	5'-	132	167	-3'
OMD2	3'-	---	GGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACA---	-biotin-5'
OMD6	3'-		CGCCTTGGCCACTCATGT	-3'
			CCTCTCGGTATCACCAGA	

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EXAMPLE 2 Capture of HIV ss DNA using a capture oligonucleotide and at least one additional oligonucleotide as the modular probe.

Methods for the identification and/or capture of HIV target nucleic acid are performed analogously to those described in Example 1 for HCV using the following modular probes:

OMD82x13 + OMD83 (13+18)

3'-TTAATTTTCGGTCC-5' + 3'-TTACCTACCGGGTTTCA-5'-biotin, or

OMD81 + OMD82 (18+18)

3'-AGGATAACTTTGACATGG-5' + 3'-TCATTTTAATTTTCGGTCC-5'-biotin

in which OMD83 and OMD82 are capture oligonucleotides.

EXAMPLE 3: Capture of sequencing products generated by the USP primer using a capture oligonucleotide and at least one additional oligonucleotide as the modular probe.

Methods for the identification and/or capture of sequencing products generated by extension of the universal sequencing primer (USP) are performed analogously to those described in Example 1 for HCV using the following modular probes:

JL-H1/USP + JL-C2/USP (9+9)

3'-GACGTCCAG-5' + 3'-CTGAGATCT-5'-biotin, or

JL-H2/USP + JL-C1/USP (13+18)

3'-GTTCTGAACGTACG-5' + 3'-GACGTCCAGCTGAGATCT-5'-biotin, or

JL-H2/USP + JL-H1/USP + JL-C2/USP (13+9+9)

3'-GTTCTGAACGTACG-5' + 3'-GACGTCCAG-5' +

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3'-CTGAGATCT-5'-biotin

in which JL-C1/USP + JL-C2/USP are capture oligonucleotides.

EXAMPLE 4 Use of modular oligonucleotides to capture HCV DNA or RNA

MATERIALS AND METHODS

Magnetic beads carrying capture oligonucleotide C1

A hepatitis C virus specific capture oligonucleotide C1 (Table 1) was covalently coupled to paramagnetic beads (Dynal, AS). The magnetic beads (10 mg/ml) were conditioned by washing twice in binding/ washing buffer (B/W) (10 mM Tris-HCl (pH 7.5), 1 mM HCl, 2 M NaCl, 1 mM β -mercaptoethanol, 0.1% Tween 20). To reduce the non-specific adsorption of nucleic acids, 1 μ g of *E.coli* tRNA (Boehringer Mannheim, Germany), was added to the beads which were then resuspended in 6X SSPE (0.9 M NaCl (pH 7.4), 60 mM NaH_2PO_4 and 7.5 mM EDTA) to a final concentration of 10 mg/ml.

Construction of recombinant hepatitis C target

Hepatitis C RNA was extracted from serum samples from infected individuals and RT-PCR carried out as described by Yun et al, 1993 (J. Med. Virol., 39, p 57-61) using the primers OU49 and OD66 (see Example 1). This generated a 324 bp fragment containing the 5'non-translated region (NTR) of HCV (nucleotides 18-341 of the HCV genome, GenBank database, Accession:M62321) which was initially sub-cloned into the pGEM[®]-T (Promega, Madison, WI, USA) vector and then inserted and cloned between the *Sph*I and *Sal*I restriction sites in the polylinker of plasmid pGEM[®]-4Z (Promega, Madison, WI, USA).

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Preparation of recombinant single strand hepatitis C DNA
Single strand DNA targets were prepared by PCR amplification of the 5'NTR of HCV cloned in pGEM®-4Z using the primers OU49 and OD66 as described in Example 1.

Solid phase (beads) hybridisation of single strand DNA
Single strand DNA target was serially diluted in a 10 fold fashion (from 10^{13} to 10^4 copies/ml HCV DNA) in a buffer containing $0.2 \mu\text{g}/\mu\text{l}$ *E. coli* tRNA. A pre-hybridisation procedure was executed by incubation of $30 \mu\text{l}$ s/s DNA at 54°C for 15 minutes in $100 \mu\text{l}$ 6X SSPE containing $1 \mu\text{g}$ *E. coli* tRNA and $0.5 \mu\text{M}$ oligonucleotide module H1-18 (Table 1). Control samples without this pre-hybridising oligonucleotide were prepared in parallel. DNA samples (with and without the pre-hybridising oligonucleotide) were then hybridised to the previously prepared magnetic beads (coupled C1) by incubating the hybridisation mix with $250 \mu\text{g}$ of beads for 1.5 hours at room temperature with constant rotation. After the hybridisation step, the beads were washed 6 times in B/W buffer (and changed to a new eppendorf tube prior to the final washing step) and resuspended in $100 \mu\text{l}$ H_2O . The DNA bound bead suspension was either used immediately in PCR or was stored at 4°C . PCR amplification was performed with $0.2 \mu\text{M}$ of the pre-hybridising oligonucleotide module (H1-18) and the upstream primer (OU 49) in a $50 \mu\text{l}$ reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl_2 , 0.2 mM dNTP's and 0.5 U of AmpliTaq® DNA polymerase (Perkin-Elmer, Foster City, CA). The mixture was overlaid with $50 \mu\text{l}$ of light mineral oil (Sigma Chemical Co., St. Louis, Mo.) and $5 \mu\text{l}$ of resuspended beads were added through this layer of mineral oil. The PCR was performed with a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Norwalk, CT) using a temperature profile of 94°C for 5 minutes, followed by 35 cycles of 94°C for 15

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seconds, 62°C for 45 seconds, 72°C for 1 minute and ending with 72°C for 10 minutes. Semi-nested PCR was performed with H1-18 and IU50 (5'-GGA ACT ACT GTC TTC ACG CAG A-3') on 5 µl of the outer PCR product using the the same cycling conditions as above except with an annealing temperaure of 55°C. PCR products were electrophoresed in a 1% agarose gel and visualised by ethidium bromide staining. During the PCR, multiple negative controls without template DNA were included. To avoid contamination, separate rooms were used for mixing the reagents, addition of sample and PCR analysis.

Preparation of recombinant hepatitis C RNA

Purified plasmid in pGEM®-4Z clone containing the 5' NTR of HCV was linearised downstream of the insert sequence by digesting 5 µg of DNA with NarI for 6 hours at 37°C. Phenol-chloroform extraction and ethanol precipitation was carried out. The resulting DNA pellet was dissolved in 50 µl diethylpyrocarbonate (DEPC) (Sigma Chemical Co., St. Louis, Mo.) treated H₂O. Transcription from the T7 promoter was performed on 1.5 µg of the linearised DNA at 37°C for 1 hour in a 50 µl reaction volume containing 30 Units T7 PNA Polymerase (Pharmacia Biotech, Uppsala, Sweden) 40 mM Tris-HCl (pH 8.0), 30 mM MgCl₂, 10 mM β-mercaptoethanol, 0.4 mM dNTP's, 5 µg BSA (RNase and DNase free, Boehringer Mannheim, Germany), 10 mM DTT and approximately 40 Units RNaguard Ribonuclease Inhibitor (Pharmacia Biotech, Uppsala, Sweden) which generated a transcript of 649 nt in length. After transcription, template DNA was fragmented by restriction digestion (with AvaI) and treatment with 8 units RNase free DNase I (Boehringer Mannheim, Germany) at 37°C for 45 minutes. Following phenol-chloroform extraction and ethanol precipitation the resulting pellet was resuspended in 50 µl DEPC treated H₂O. The *in vitro* transcribed RNA was then analysed by gel electrophoresis and quantified by measuring OD₂₆₀. The OD₂₆₀/OD₂₈₀ ratio of the obtained RNA

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preparation was 1.87 ± 0.1 . A 5 and a 10 fold dilution series of RNA was then made in $10 \text{ ng}/\mu\text{l}$ *Escherichia coli* tRNA.

Biosensor analysis of recombinant hepatitis C RNA

Biosensor experiments were performed using a BIAcore 2000 instrument as described in Example 1, using C1 (Table 1) as the capture module with the exception that the injection volume was increased to $60 \mu\text{l}$ which was injected at a flow rate of $30 \mu\text{l}/\text{minute}$. Six microlitres of RNA transcript was pre-hybridised to $0.5 \mu\text{M}$ oligonucleotide module (H1-18) in $100 \mu\text{l}$ 6X SSPE by incubation at 54°C for 15 minutes followed by cooling to room temperature. Forty microliters of this hybridization mix was injected over the immobilised capture oligonucleotide (C1) at a flow of $2 \mu\text{l}/\text{minute}$. Samples with no pre-hybridizing oligonucleotide were treated in exactly the same manner.

Solid phase (beads) hybridisation and detection of recombinant hepatitis C RNA

The procedure for capturing of RNA on beads is the same as that outlined for the capture of DNA, namely pre-hybridisation of the oligonucleotide module to $30 \mu\text{l}$ RNA at 54°C for 15 minutes followed by capture on magnetic beads (linked to the HCV specific capture probe C1) by rotation at room temperature for 1.5 hours. RNA was serially diluted in a 5 fold fashion (from 5×10^7 to 1.6×10^4 copies/ml) in $0.2 \mu\text{g}/\mu\text{l}$ *E. coli* tRNA. The beads with the captured mRNA were washed 4 times in B/W buffer and twice in cold RT-PCR buffer (they were changed to a new eppendorf tube prior to the final washing step) and resuspended in $100 \mu\text{l}$ of DEPC treated H_2O before being used for RT-PCR. If the bead suspension was not used immediately for RT-PCR it was stored at -70°C . During transcription and RNA capture, all glassware and solutions (with the exception of Tris buffers) were DEPC

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treated to avoid possible contamination with RNase. Reverse transcription and outer PCR was performed in a one tube assay. Reverse transcription was carried out on 5 μ l of resuspended beads at 37°C for 1 hour (with continuous rotation) using 0.5 Units of MMLV Reverse Transcriptase, followed by PCR amplification with 2 Units Ampli Taq Gold® (Perkin-Elmer, Foster City, CA) in a total reaction volume of 50 μ l. The reaction conditions were the same as those described above for the outer PCR with the addition of a pre-heating step at 94°C for 12 minutes to activate Ampli Taq Gold®. Four micrograms of *E. coli* tRNA was also included to prevent inhibition of Taq polymerase activity by reverse transcriptase [Sellner et al., 1992, Nucl. Acids Res., 20, p 1487-1490]. Positive and negative controls were included as well as a no reverse transcriptase control. Five microlitres of the outer PCR mix was used in the semi-nested inner PCR with the pre-hybridising oligonucleotide module (H1-18) and upstream primer IU50.

Modular assisted capture of hepatitis C in clinical samples using beads as the solid phase

Serum samples from HCV-infected patients stored at -20°C were used. The samples were genotyped (Yun et al, 1993, supra) and quantitated using Amplicor HCV Monitor Test (Roche Molecular Systems). Initially, 0.5 μ M oligonucleotide module was pre-hybridised to 100 μ l serum sample in 1 ml 6X SSPE containing 1 μ g *E. coli* tRNA and 500 μ l solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M β -mercaptoethanol) by heating at 60°C for 10 minutes followed by rotation at room temperature for 45 minutes. Every 6th sample was a non-HCV serum negative control. Beads (250 μ g) covalently coupled to C1, prepared as described above, were then added to this hybridisation mix and rotated at room temperature for 1 hour to facilitate capture. The beads were then washed 4 times

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in 100 μ l B/W buffer and twice in 100 μ l PCR buffer. The beads were resuspended in 20 μ l H₂O, heated at 70°C for 3 minutes and placed immediately on ice. RT-PCR was carried out using 10 μ l of bead suspension as described by Yun et al, (1993, supra).

RESULTS

Hybridisation onto magnetic beads of single stranded DNA
In order to investigate the use of modular probes in magnetic bead mediated sample preparation of hepatitis C virus a model system was established, as schematically outlined in Figure 7. The solid support for these experiments were beads with a covalently coupled 18 mer probe (C1) complementary to the virus target. An 18 mer long oligonucleotide module (H1-18) was designed and synthesised to anneal adjacent to the immobilised capture probe. Single strand DNA corresponding to the 5' non translated region (NTR) of hepatitis C was prepared by *in vitro* amplification and alkali strand separation according to the solid phase sequencing procedure. Quantified single strand DNA templates were 10 fold serially diluted. A pre-hybridisation step at 54°C for 15 minutes involving the oligonucleotide module and serially diluted templates was performed. The hybridisation mixtures were subsequently incubated with magnetic beads for solid phase capture at room temperature for 90 minutes. After incubation the beads were washed and transferred to PCR tubes containing reagents and primers for single amplification of the hepatitis C target region. Incubation of control samples without the oligonucleotide module in the pre-hybridisation step were performed in parallel. If amplification was successful a fragment of approximately 320 bp was expected. The results after the amplification are depicted in Figure 8. In the top panel which corresponds to samples prepared with an oligonucleotide module, an amplified fragment can

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be observed down to the dilution step containing approximately 10^4 starting molecules, while without the oligonucleotide module an approximately 10-fold lower sensitivity is achieved (a weak fragment is observed in the dilution step corresponding to 10^5 starting molecules). This indicates the benefit of using a modular probe in bead assisted capture.

Hybridisation of RNA analysed by BIAcore

The previous set of experiments have focused on the use of DNA targets corresponding to the positive strand of the hepatitis C virus RNA genome. Therefore to allow for a more direct comparison with true samples, *in vitro* transcribed RNA samples were generated. After *in vitro* transcription of a linearised plasmid construct containing the target region, the 649 nt long transcript was extracted and quantified. The RNA produced was used as target in a similar set-up as previous BIAcore analysis of single stranded DNA. Thus the RNA was prehybridized with oligonucleotide module (500 nmol) as used above (H1-18, 18 mer) and then passed over the immobilised capture oligonucleotide (C1, biotinylated 18 mer) on the chip surface. A control sample without the oligonucleotide module was processed in parallel. These two samples were also passed over a chip surface without any immobilised capture oligonucleotide which acted as a blank control. The resulting data is presented as an overlay plot for the two subtracted samples in Figure 9. The data clearly indicates that significantly more target RNA is captured when a modular probe has been employed. It is also important to note that the reactions have not reached saturation during the injection pulse (20 min) and therefore it is likely that the absolute differences are even higher.

Hybridisation and detection of RNA onto magnetic beads

As a result of the successful BIAcore analysis the model

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system was also evaluated on magnetic beads with a covalently bound capture oligonucleotide (C1). To facilitate comparison, the template RNA was 10-fold serially diluted as described previously for DNA templates. The dilutions were then incubated at 54°C for 15 minutes with the oligonucleotide module 18 mer probe (H1-18) followed by a further incubation with magnetic beads at room temperature for 90 minutes. Control samples without H1-18 were processed in parallel. After a washing step a one tube RT-PCR was performed on the samples. The results are presented in Figure 10 and show a weak fragment in the dilution corresponding to approximately 10^4 starting RNA molecules, while without the oligonucleotide module an approximately 10-fold lower sensitivity is achieved. To further investigate the quantitative differences a more narrow dilution series (5-fold) was used in an RT-nested PCR experiment. Nested PCR will allow for a comparison at the PCR plateau level at which all dilutions have reached saturation irrespective of the number of starting copies. Figure 11 shows that with an oligonucleotide module probe 140 RNA starting copies can be detected, while without the oligonucleotide module probe 700 copies are required for detection. These results are in complete agreement with the results obtained with single stranded DNA templates.

Detection of hepatitis C in clinical samples using module assisted capture

The encouraging results with our two model systems based on either DNA or RNA targets indicated that modular probes improved capture onto either a chip surface or a solid particle. This lead us to evaluate the approach on clinical samples containing hepatitis C virus. First we analysed two HCV positive samples (using a similar approach to that outlined above) by serially diluting the samples 5-fold followed by incubation in a denaturing solution containing 500 nmol of the oligonucleotide

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module at 60°C for 10 minutes. These were then incubated at room temperature for 45 minutes before addition of beads with covalently coupled capture probe and a further incubation at room temperature for 60 minutes. After washing, the beads were directly transferred into RT-PCR tubes as described above. Figure 12 shows one of the two samples with and without a oligonucleotide module and confirms the same trend as previously demonstrated i.e. that inclusion of an oligonucleotide module improves the capture performance. Also upon a further amplification of these two serially diluted samples with inner primers an approximate absolute value is obtained which indicates up to a 25-fold higher sensitivity with the oligonucleotide module (data not shown).

Finally, a total of 19 clinical samples were then analysed using the described approach. All of these had previously been quantified by a commercial test (Amplicor HCV Monitor Test, Roche). In 5 of these 14 samples a comparison with and without oligonucleotide module was also possible. The results are depicted in Table 3 and show a good correlation between the commercial test and the module assisted capture for all virus titers. Interestingly, in one of the five samples that were compared, viral capture failed when the oligonucleotide module was omitted. This confirms the trend seen with the model systems and truly shows that the prehybridization step also increases the sensitivity of detection for clinical samples.

DISCUSSION

This study shows the utility of modular oligonucleotides in the capture of single stranded templates. Interestingly, it is not limited only to short fragments as employed in our model system but also complete hepatitis C genomes are more efficiently captured. No

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difference can be observed between DNA and RNA targets which could have been expected due to their different chemical structures. In contrast identical capture patterns were displayed when tested with and without an oligonucleotide module.

Preliminary studies have also suggested that the protocol for viral capture could be shortened by combining the prehybridization, sample lysis (in guanidium thiocyanate) and bead capture in a single step. Thereby only a simple washing step is required prior to RT-PCR making the system very attractive for automated approaches.

Table 3 - Summary of the results using clinical samples

Sample	Genotype	Quantitative determination (copies/ml)	Routine HCV procedure	Solid Phase approach
1	1b	2.5×10^6	+	+
2	2b	2.5×10^6	+	+
3	1b	2.5×10^6	+	+
4	2b+1b	5.0×10^5	+	+
5	1a	nd	+	+
6	control			-
7	3a	1.0×10^5	+	+
8	1a+1b	1.0×10^5	+	+
9	3a	1.0×10^5	+	+
10	2b+1b	5.0×10^5	+	+
11	1a+1b	5.0×10^5	+	+
12	control			-
13	1b	1.0×10^5	+	+
14	3a	2.0×10^4	+	+
15	1a	5.0×10^5	+	+
16	2b	5.0×10^5	+	+
17	1b	2.5×10^6	+	+
18	control			-
19	2b	5.0×10^5	+	+

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20	1a	1.0×10^5	+	+
21	3a	2.5×10^6	+	+
22	1b	1.0×10^5	+	+
23	control			-

EXAMPLE 5 Oligonucleotide module assisted capture of HIV-1 virus

Experiments on a model system consisting of a cloned fragment of the HIV-1 genome, the pol region (Table 4) are described. The pol region is a frequently used target in different diagnostic systems for detection and quantification of HIV-1 virus.

MATERIALS AND METHODS

Construction of recombinant HIV target and preparation of single strand HIV DNA

PCR was carried out on the proviral HIV-1_{MN} strain (Myers et al, 1991, Human Retrovirus and AIDS 1991, Los Alamos National Laboratory, Los Alamos, New Mexico) using POL specific primers JA 79 and TV 84 (Table 4). This generated a 378 bp fragment which was cloned into the pGEM®-T vector. Single strand DNA was prepared by PCR amplification of this cloned POL gene using the vector specific primers RIT 28 and RIT 29. The resulting biotinylated 800 bp fragment was subjected to strand specific elution as described in Example 1.

Biosensor analysis of recombinant HIV DNA

A HIV specific biotinylated oligonucleotide (OMD82, see Example 2) was immobilised on the sensor chip followed by injection of 40 µl of single stranded HIV DNA prehybridised to OMD 81 (as described for the HCV target). A control sample with no oligonucleotide module was run in parallel. The target sequence in this case

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is 5'-TCCTATTGAAACTGTACCAGTAAAATTAAAGCCAGG-3' which is nucleotides 473-509 of the HIV-1 genome.

Table 4 - PCR Primers

JA79 (pol)

5'-ACAGGAGCAGATGATACAGTATTAG-3'

TV 84 (pol)

5'-GACATTCGAATTCCCTTCCTTTTCCATTTCTGTAC-3'

RIT 28 (vector)

5'-AAAGGGGGATGTGTGCTGCAAGGCG-3'

RIT 29 (vector)

5'-biotin-GCTTCCGGCTCGTATGTTGTGTG-3'

RESULTS

Single strand DNA pol targets were generated in a similar manner as the hepatitis C case; ie. strand specific elution of biotinylated PCR amplicons using streptavidin coated magnetic beads. The resulting single strand DNA targets were injected over a sensor chip surface containing a complementary probe sequence and the interaction was measured in real time by the biosensor system. The experiments were performed with and without an oligonucleotide module. The results (Figure 13) show again that modular probes do enhance capture as determined by the overlay plot from the biosensor experiment.

SEQUENCE LISTING

GENERAL INFORMATION

APPLICANT: DYNAL AS

TITLE OF INVENTION: The Use of Modular Oligonucleotides as
Probes or Primers in Nucleic Acid Based Assay

NUMBER OF SEQUENCES: 36

CORRESPONDENCE ADDRESS: Kirby Eades Gale Baker
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CANADA

COMPUTER READABLE FORM:

MEDIUM TYPE: Floppy disk

COMPUTER: IBM PC compatible

OPERATING SYSTEM: PC-DOS/MS-DOS

SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

CURRENT APPLICATION DATA:

APPLICATION NUMBER: 2,266,749

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APPLICATION NUMBER: GB 9620075.3

FILING DATE: 26-SEP-1996

CLASSIFICATION:

PATENT AGENT INFORMATION:

NAME: Andrew Bauer-Moore

REFERENCE NUMBER: 42979-NP

INFORMATION FOR SEQ ID NO: 1:

SEQUENCE CHARACTERISTICS:

LENGTH: 18 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - H1-18"

SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCTCCCGGGG CACTCGCA

INFORMATION FOR SEQ ID NO: 2:

SEQUENCE CHARACTERISTICS:

LENGTH: 18 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - C1"

SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGTGCACGGT CTACGAGA

18

INFORMATION FOR SEQ ID NO: 3:

SEQUENCE CHARACTERISTICS:

LENGTH: 11 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - H2"

SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTCCCGGGGC A

11

INFORMATION FOR SEQ ID NO: 4:

SEQUENCE CHARACTERISTICS:

LENGTH: 15 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - H1-15"

SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCTCCCGGGG CACTC

15

INFORMATION FOR SEQ ID NO: 5:

SEQUENCE CHARACTERISTICS:

LENGTH: 13 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - H1-13"

SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCTCCCGGGG CAC

13

INFORMATION FOR SEQ ID NO: 6:

SEQUENCE CHARACTERISTICS:

LENGTH: 11 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - H1-11"

SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCTCCCGGGG C

11

INFORMATION FOR SEQ ID NO: 7:

SEQUENCE CHARACTERISTICS:

LENGTH: 9 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - H8"

SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCACTCGCA

9

INFORMATION FOR SEQ ID NO: 8:

SEQUENCE CHARACTERISTICS:

LENGTH: 9 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - H1-9"

SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCTCCCGGG

9

INFORMATION FOR SEQ ID NO: 9:

SEQUENCE CHARACTERISTICS:

LENGTH: 9 base pairs

TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid
DESCRIPTION: /desc = "synthetic oligonucleotide - H4"
SEQUENCE DESCRIPTION: SEQ ID NO: 9:
TCTACGAGA

9

INFORMATION FOR SEQ ID NO: 10:
SEQUENCE CHARACTERISTICS:
LENGTH: 9 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid
DESCRIPTION: /desc = "synthetic oligonucleotide - C2"
SEQUENCE DESCRIPTION: SEQ ID NO: 10:
GGTGCACGG

9

INFORMATION FOR SEQ ID NO: 11:
SEQUENCE CHARACTERISTICS:
LENGTH: 9 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid
DESCRIPTION: /desc = "synthetic oligonucleotide - H3"
SEQUENCE DESCRIPTION: SEQ ID NO: 11:
CTCCCGGGG

9

INFORMATION FOR SEQ ID NO: 12:
SEQUENCE CHARACTERISTICS:
LENGTH: 9 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid
DESCRIPTION: /desc = "synthetic oligonucleotide - H5"
SEQUENCE DESCRIPTION: SEQ ID NO: 12:
CTACGAGAC

9

INFORMATION FOR SEQ ID NO: 13:

SEQUENCE CHARACTERISTICS:

LENGTH: 18 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - OMD6"

SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGACCACTAT GGCTCTCC

18

INFORMATION FOR SEQ ID NO: 14:

SEQUENCE CHARACTERISTICS:

LENGTH: 18 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - OMD2"

SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TGTACTCACC GGTTCGC

18

INFORMATION FOR SEQ ID NO: 15:

SEQUENCE CHARACTERISTICS:

LENGTH: 13 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - OMD82x13"

SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCTGGCTTTA ATT

13

INFORMATION FOR SEQ ID NO: 16:

SEQUENCE CHARACTERISTICS:

LENGTH: 18 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - OMD83"

SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ACTTTTGGGC CATCCATT

18

INFORMATION FOR SEQ ID NO: 17:

SEQUENCE CHARACTERISTICS:

LENGTH: 18 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - OMD81"

SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGTACAGTTT CAATAGGA

18

INFORMATION FOR SEQ ID NO: 18:

SEQUENCE CHARACTERISTICS:

LENGTH: 18 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - OMD82"

SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CCTGGCTTTA ATTTTACT

18

INFORMATION FOR SEQ ID NO: 19:

SEQUENCE CHARACTERISTICS:

LENGTH: 9 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - JL-H1/USP"

SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GACCTGCAG

9

INFORMATION FOR SEQ ID NO: 20:

SEQUENCE CHARACTERISTICS:

LENGTH: 9 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - JL-C2/USP"

SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TCTAGAGTC

9

INFORMATION FOR SEQ ID NO: 21:

SEQUENCE CHARACTERISTICS:

LENGTH: 13 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - JL-H2/USP"

SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GCATGCAAGC TTG

13

INFORMATION FOR SEQ ID NO: 22:

SEQUENCE CHARACTERISTICS:

LENGTH: 18 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - JL-C1/USP"

SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TCTAGAGTCG ACCTGCAG

18

INFORMATION FOR SEQ ID NO: 23:

SEQUENCE CHARACTERISTICS:

LENGTH: 21 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - OU49"

SEQUENCE DESCRIPTION: SEQ ID NO: 23:
GGCGACACTC CACCATGAAT C

21

INFORMATION FOR SEQ ID NO: 24:

SEQUENCE CHARACTERISTICS:

LENGTH: 20 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - OD66"

SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GGTGCACGGT CTACGAGACC

20

INFORMATION FOR SEQ ID NO: 25:

SEQUENCE CHARACTERISTICS:

LENGTH: 27 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - C3"

SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GGTGCACGGT CTACGAGACC TCCCGGG

27

INFORMATION FOR SEQ ID NO: 26:

SEQUENCE CHARACTERISTICS:

LENGTH: 36 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - C4"

SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GGTGCACGGT CTACGAGACC TCCCGGGGCA CTCGCA

36

INFORMATION FOR SEQ ID NO: 27:

SEQUENCE CHARACTERISTICS:

LENGTH: 28 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid
DESCRIPTION: /desc = "synthetic oligonucleotide - C1x10A"
SEQUENCE DESCRIPTION: SEQ ID NO: 27:
AAAAAAAAAA GGTGCACGGT CTACGAGA 28

INFORMATION FOR SEQ ID NO: 28:
SEQUENCE CHARACTERISTICS:
LENGTH: 18 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid
DESCRIPTION: /desc = "synthetic oligonucleotide - H6"
SEQUENCE DESCRIPTION: SEQ ID NO: 28:
TCTACGAGAC CTCCCGGG 18

INFORMATION FOR SEQ ID NO: 29:
SEQUENCE CHARACTERISTICS:
LENGTH: 15 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid
DESCRIPTION: /desc = "synthetic oligonucleotide - H7"
SEQUENCE DESCRIPTION: SEQ ID NO: 29:
AGCACCTAT CAGGC 15

INFORMATION FOR SEQ ID NO: 30:
SEQUENCE CHARACTERISTICS:
LENGTH: 5 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: other nucleic acid
DESCRIPTION: /desc = "synthetic oligonucleotide - H1-5"
SEQUENCE DESCRIPTION: SEQ ID NO: 30:
CCTCC 5

INFORMATION FOR SEQ ID NO: 31:

SEQUENCE CHARACTERISTICS:

LENGTH: 36 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: DNA (genomic)

ORIGINAL SOURCE:

ORGANISM: Hepatitis C virus

SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GGAGAGCCAT AGTGGTCTGC GGAACCGGTG AGTACA

36

INFORMATION FOR SEQ ID NO: 32:

SEQUENCE CHARACTERISTICS:

LENGTH: 36 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: DNA (genomic)

ORIGINAL SOURCE:

ORGANISM: Human immunodeficiency virus

SEQUENCE DESCRIPTION: SEQ ID NO: 32:

TCCTATTGAA ACTGTACCAG TAAAATTAAA GCCAGG

36

INFORMATION FOR SEQ ID NO: 33:

SEQUENCE CHARACTERISTICS:

LENGTH: 25 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - JA79"

SEQUENCE DESCRIPTION: SEQ ID NO: 33:

ACAGGAGCAG ATGATACAGT ATTAG

25

INFORMATION FOR SEQ ID NO: 34:

SEQUENCE CHARACTERISTICS:

LENGTH: 35 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - TV84"

SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GACATTCGAA TTCCCTTCCT TTTCCATTTT TGTAC

35

INFORMATION FOR SEQ ID NO: 35:

SEQUENCE CHARACTERISTICS:

LENGTH: 25 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - RIT28"

SEQUENCE DESCRIPTION: SEQ ID NO: 35:

AAAGGGGGAT GTGTGCTGCA AGGCG

25

INFORMATION FOR SEQ ID NO: 36:

SEQUENCE CHARACTERISTICS:

LENGTH: 23 base pairs

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

DESCRIPTION: /desc = "synthetic oligonucleotide - RIT29"

SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GCTTCCGGCT CGTATGTTGT GTG

23

THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of detecting and/or isolating a target nucleic acid molecule in a sample, wherein said method comprises at least the steps of binding a complementary modular oligonucleotide of two or three modules to directly adjacent stretches of said target nucleic acid molecule in said sample, and detecting and/or isolating the target nucleic acid molecule bound to said oligonucleotide, wherein each module has $>5 \leq 13$ nucleotides, wherein said modular oligonucleotide exhibits improved binding relative to a single composite oligonucleotide complementary to the region of the target molecule spanned by the modular oligonucleotide, wherein at least one capture module is immobilized or has means for immobilization, and wherein in methods of isolation the capture module allows said isolation.
2. A method as claimed in claim 1 wherein each module has $\geq 9 \leq 13$ nucleotides.
3. A method as claimed in claim 1 wherein each module has $>5 \leq 9$ nucleotides.
4. A method as claimed in claim 1 wherein each module has 9, 11 or 13 nucleotides.
5. A method as claimed in any one of claims 1 to 4 wherein said modular oligonucleotide consists of a total of at least 18 nucleotides.

6. A method as claimed in any one of claims 1 to 5 wherein said modular oligonucleotide consists of the capture module and modulating modules and said modulating modules are added to, or contacted with, a sample containing the target nucleic acid molecules prior to the addition of the free or immobilized capture module.

7. A method as claimed in any one of claims 1 to 6 wherein immobilization is via the streptavidin:biotin binding system.

8. A method as claimed in any one of claims 1 to 7 wherein at least one module is labelled.

9. A method as claimed in any one of claims 1 to 8 comprising the steps of:

1) contacting the sample containing the target nucleic acid with all modules of the modular oligonucleotide;

2) binding said modules to said target nucleic acid molecule by hybridization;

3) addition of a solid support and attachment of at least one of said modules provided with a means for immobilization to said solid support;

4) separating target nucleic acid bound to said solid support;

5) washing said solid support;

6) amplification of said target nucleic acid; and

7) assessing the presence or amount of amplified nucleic acid.

10. A method as claimed in any one of claims 1 to 9 wherein the extent of binding is determined by the change in refractive index at a sensor surface.

11. A method as claimed in any one of claims 1 to 10 for detecting and/or isolating a HCV nucleic acid, wherein said modular oligonucleotide comprises one of the following nucleotide sequences:

3'-CACGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5';

or

3'-CGGGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5';

or

3'-GGGCCCTCC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5';

or

3'-GGGGCCCTC-5' + 3'-CAGAGCATC-5' + 3'-GGCACGTGG-5';

or

3'-GGGGCCCTC-5' + 3'-AGAGCATCT-5' + 3'-GGCACGTGG-5';

or analogs or derivatives thereof in which nucleotide bases are modified or derivatised.

12. A method as claimed in any one of claims 1 to 10 for detecting and/or isolating primer extension products.

13. A method as claimed in claim 12 for detecting and/or isolating sequencing products.

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14. A method as claimed in claim 12 or 13 wherein said modular oligonucleotide comprises one of the following nucleotide sequences:

3'-GACGTCCAG-5' + 3'-CTGAGATCT-5';

or

3'-GTTCGAACGTACG-5' + 3'-GACGTCCAG-5' + 3'-CTGAGATCT-5',

or analogs or derivatives thereof in which nucleotide bases are modified or derivatised.

15. A method as claimed in claim 11 or 14 wherein the last listed module of the modular oligonucleotide is the capture module.

16. A modular oligonucleotide as defined in claim 11 or 14.

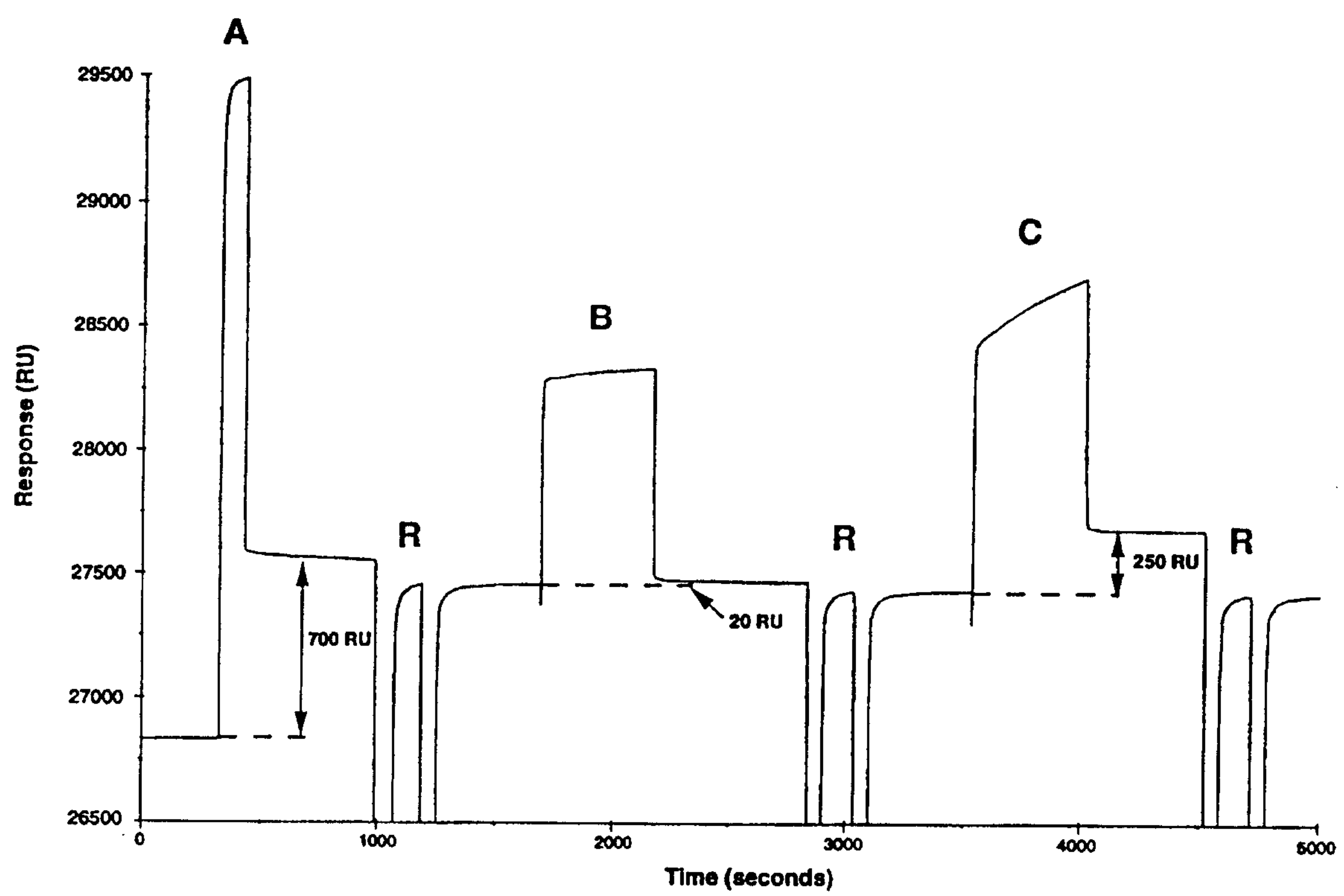
17. Use of a modular oligonucleotide as defined in claim 16 in a method as defined in any one of claims 1 to 15.

18. A kit for performing the method as defined in any one of claims 1 to 15, comprising at least the following:

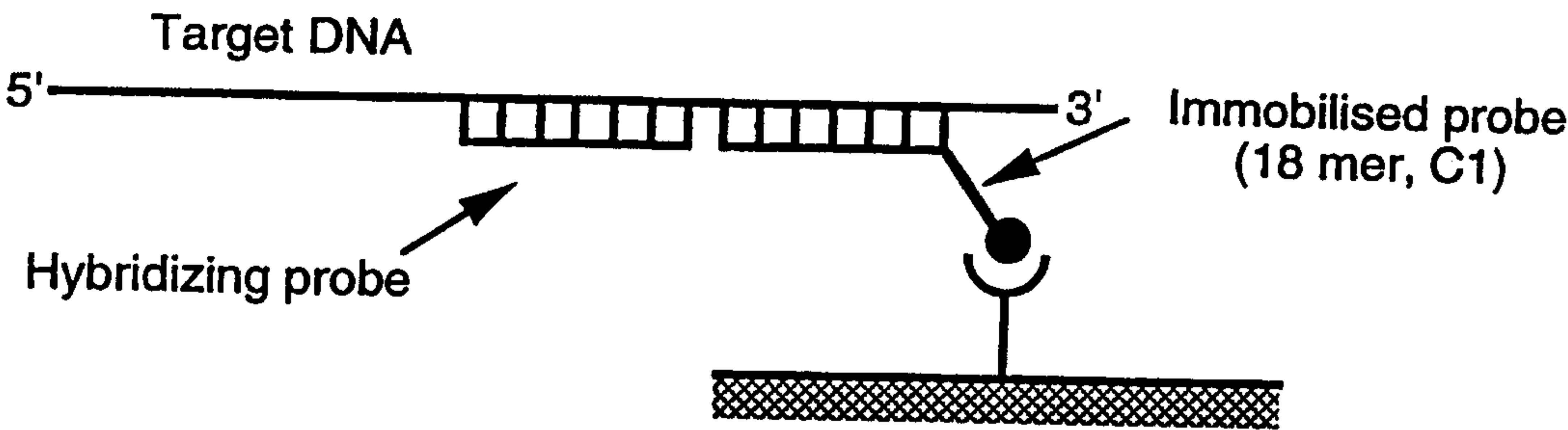
a modular oligonucleotide as defined in claim 11 or 14 and instructions for use.

19. The kit of claim 18, wherein at least one of the modules is labelled to allow detection of the target nucleic acid.

1 / 13

Figure 1

A



B

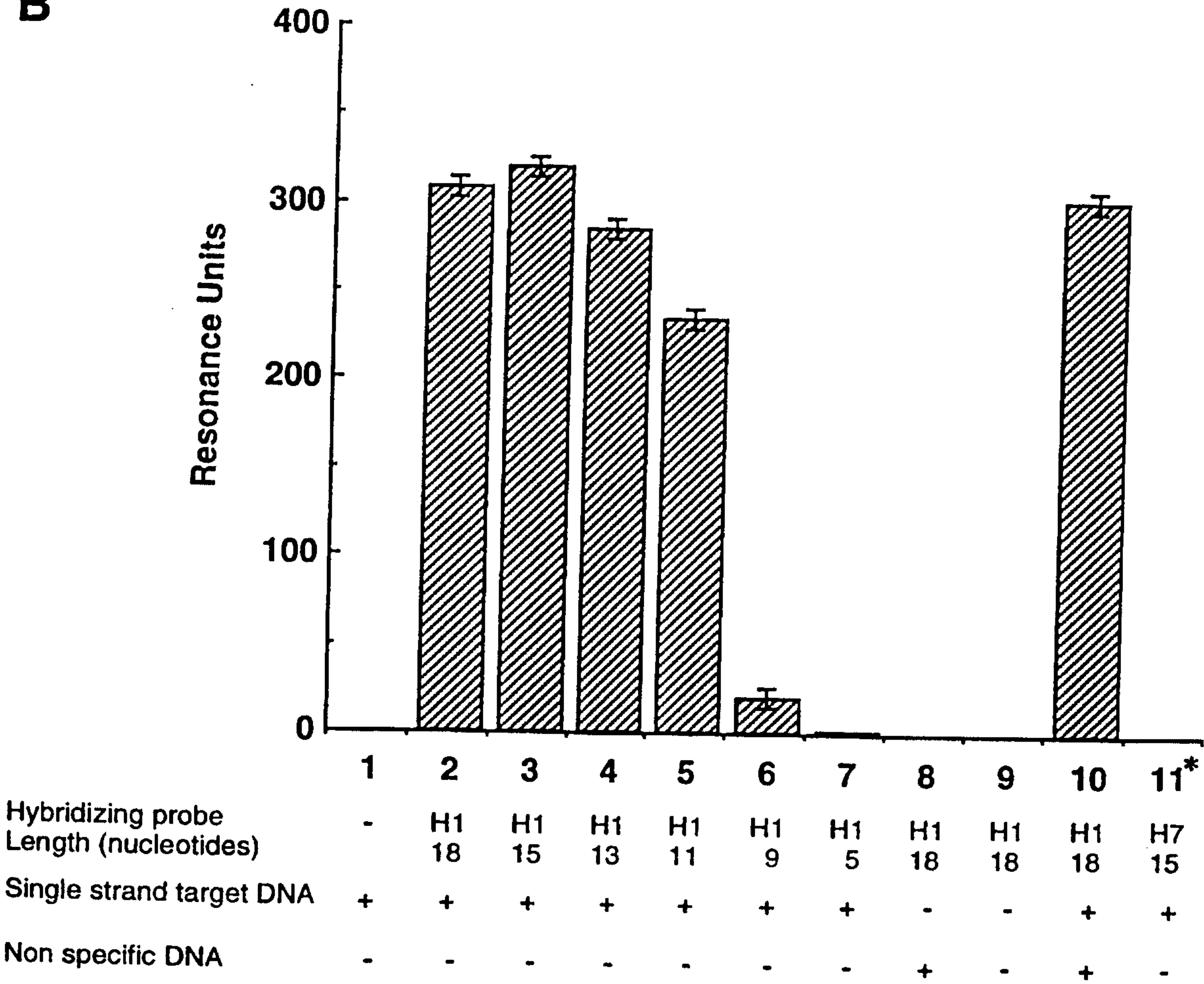
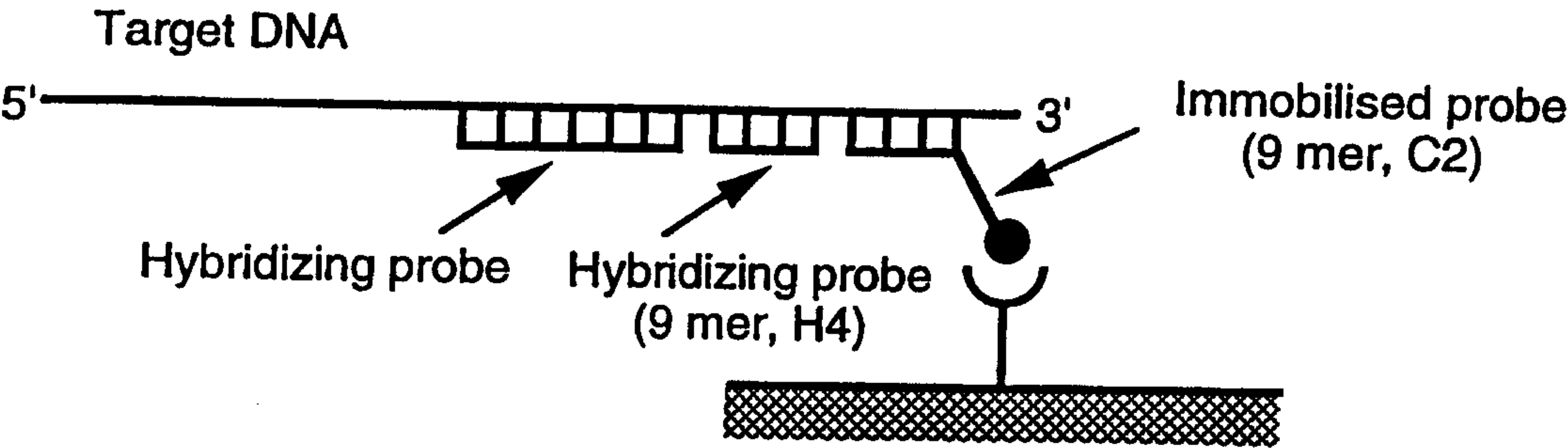


Figure 2

A



B

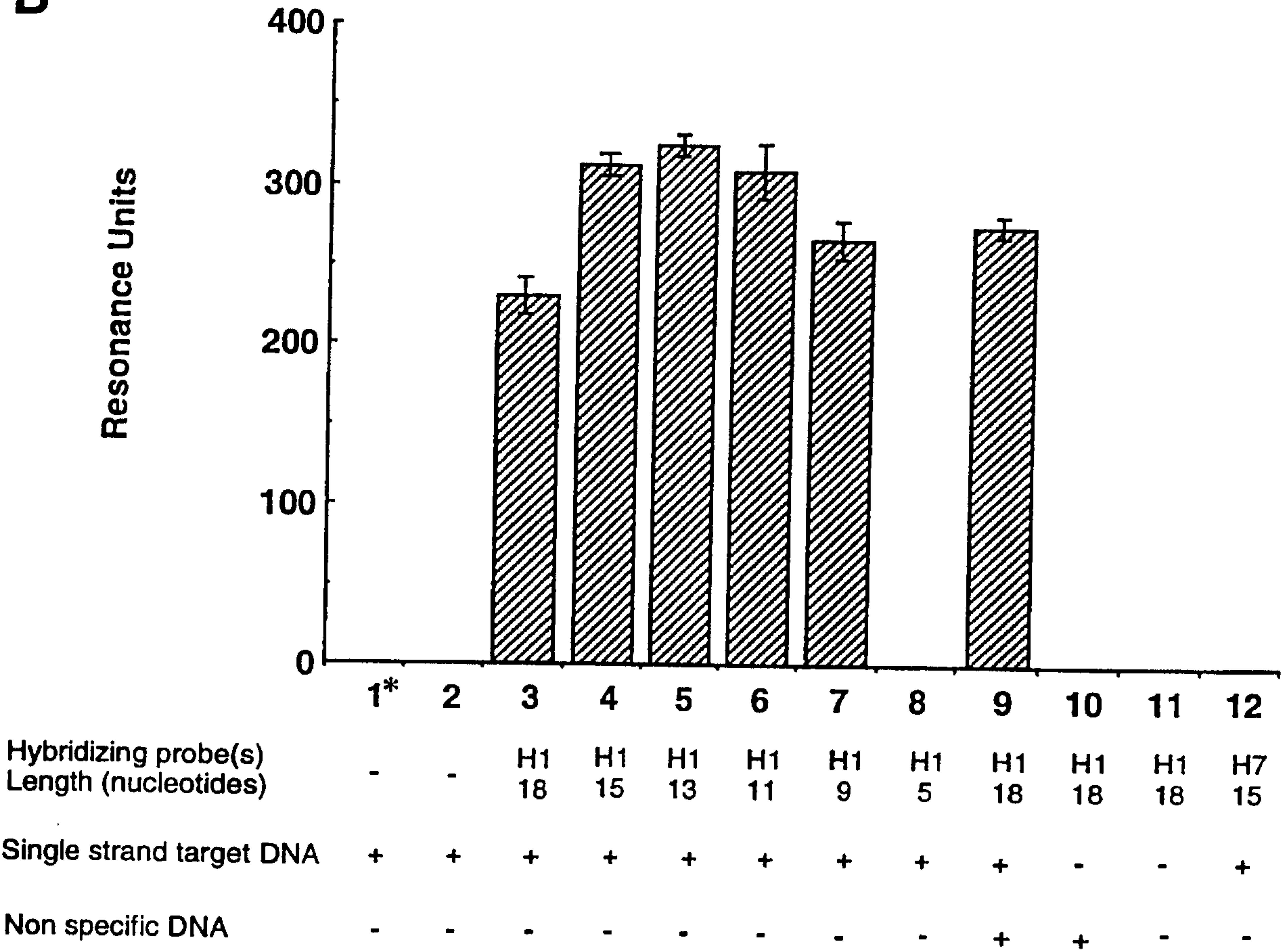


Figure 3

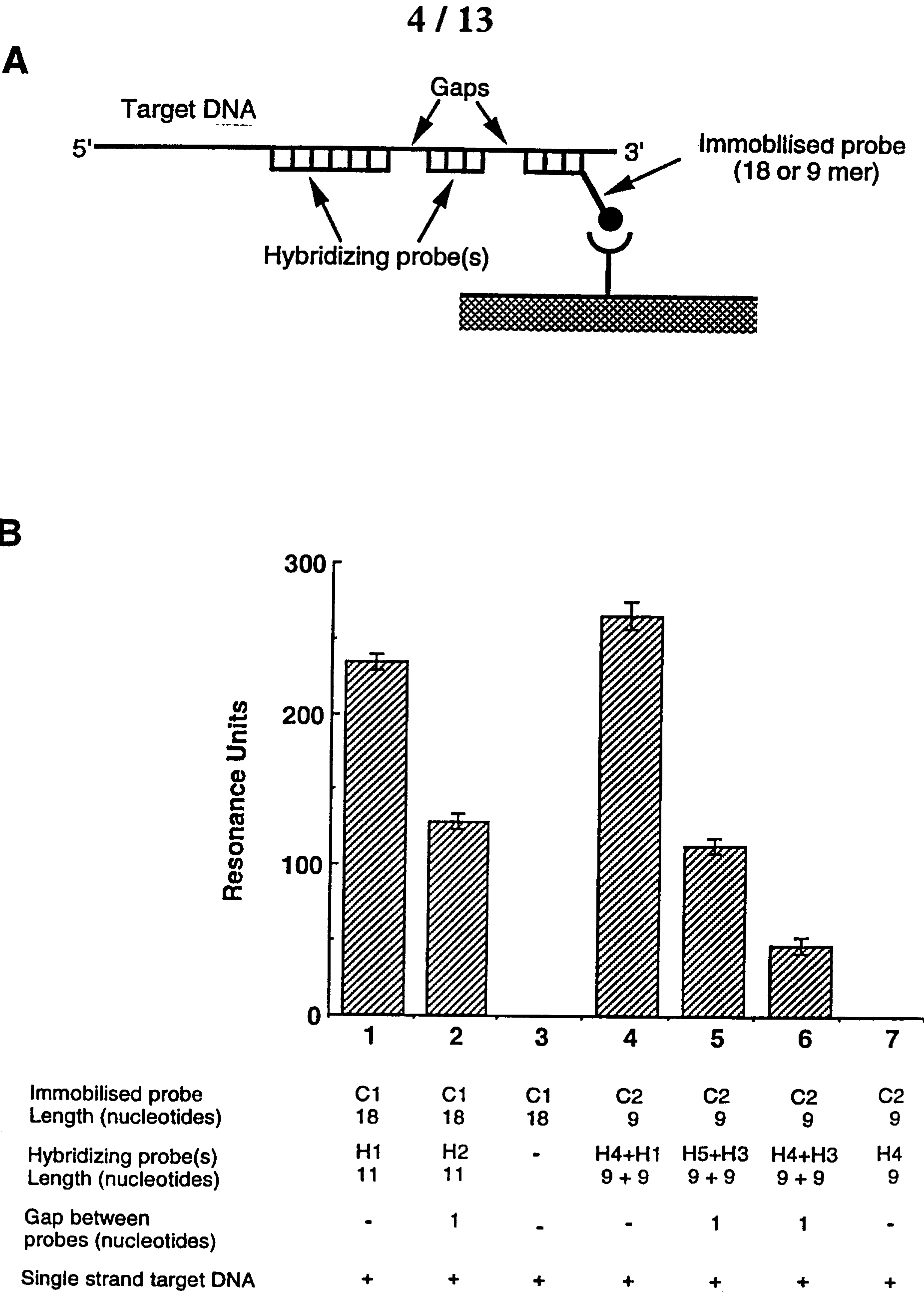


Figure 4

5 / 13

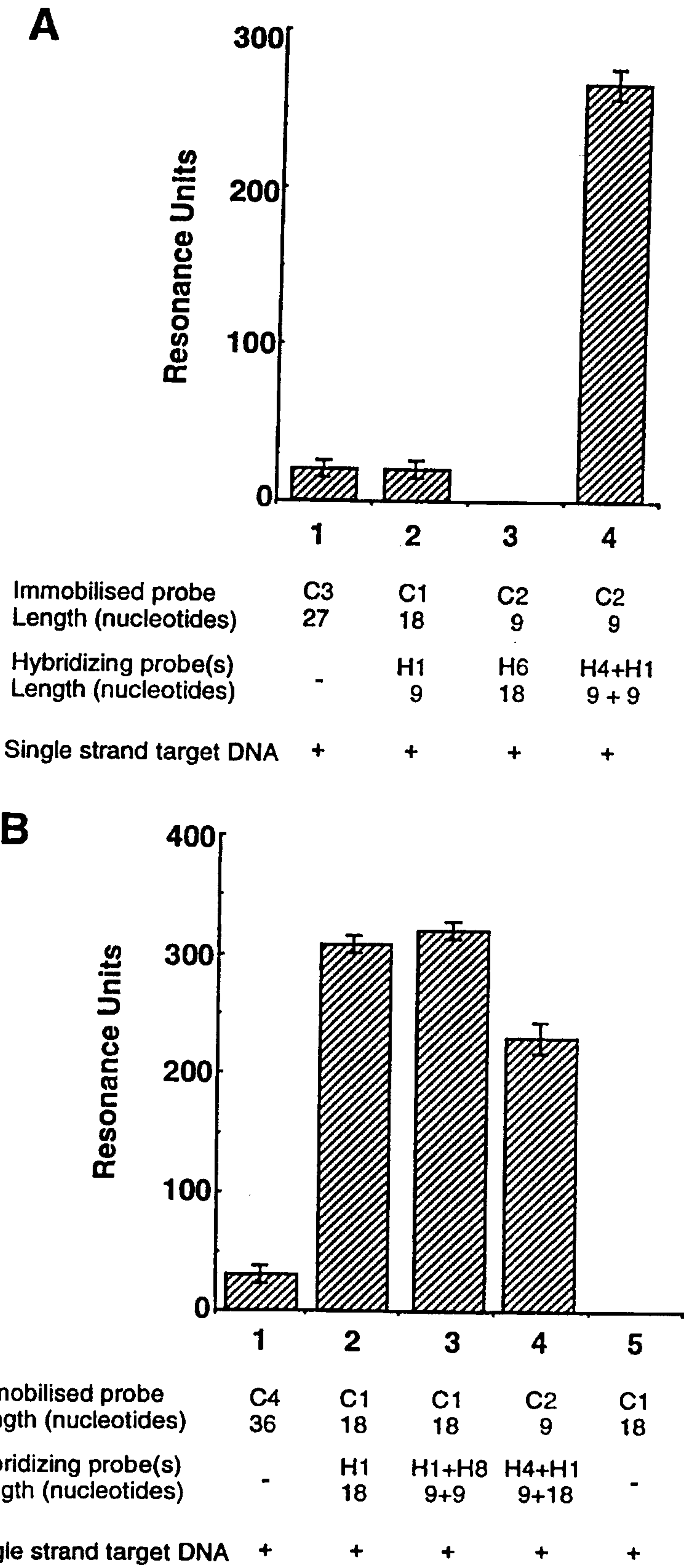
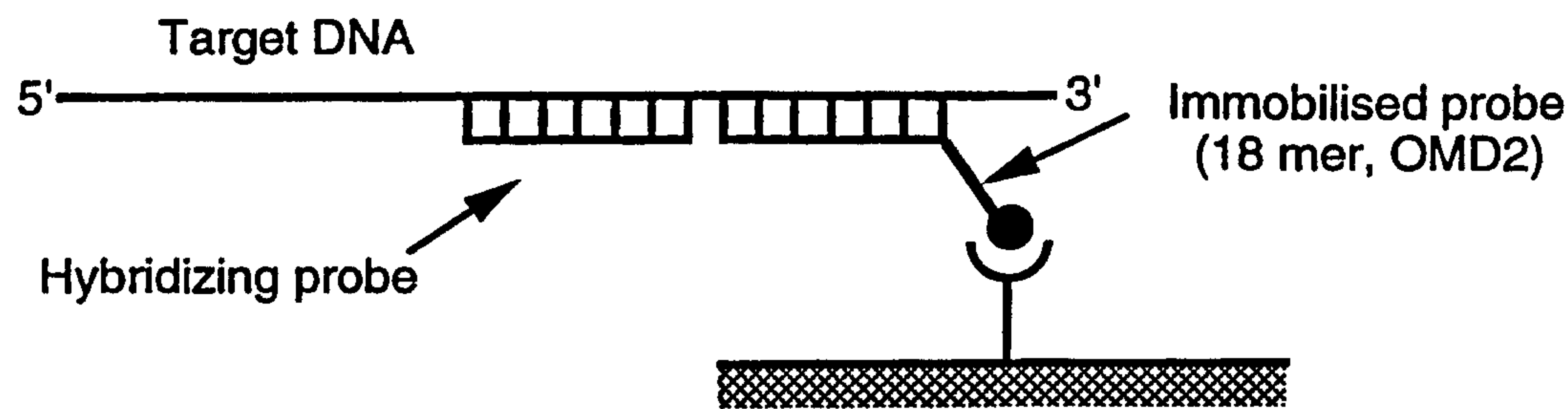


Figure 5

B



C

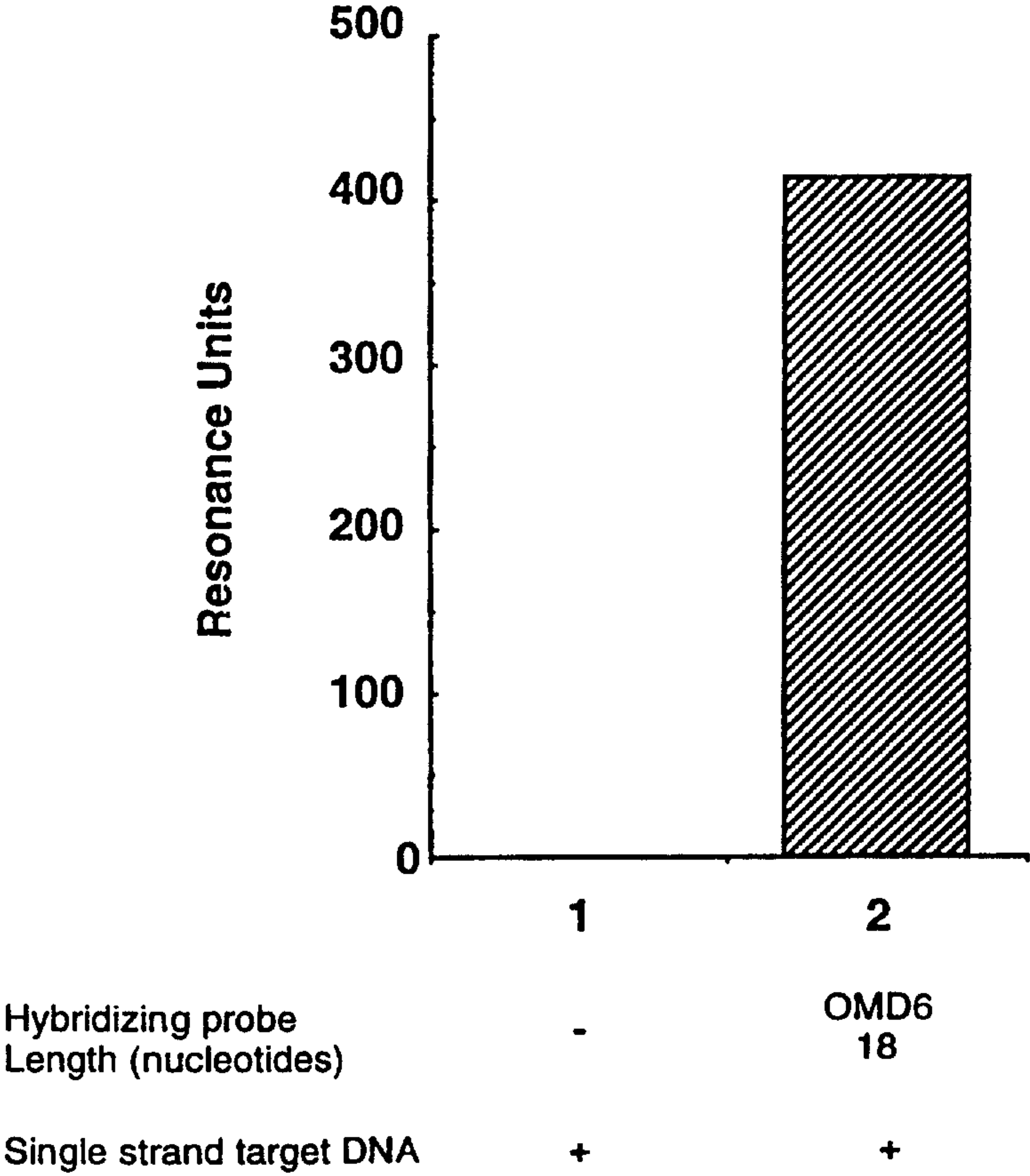
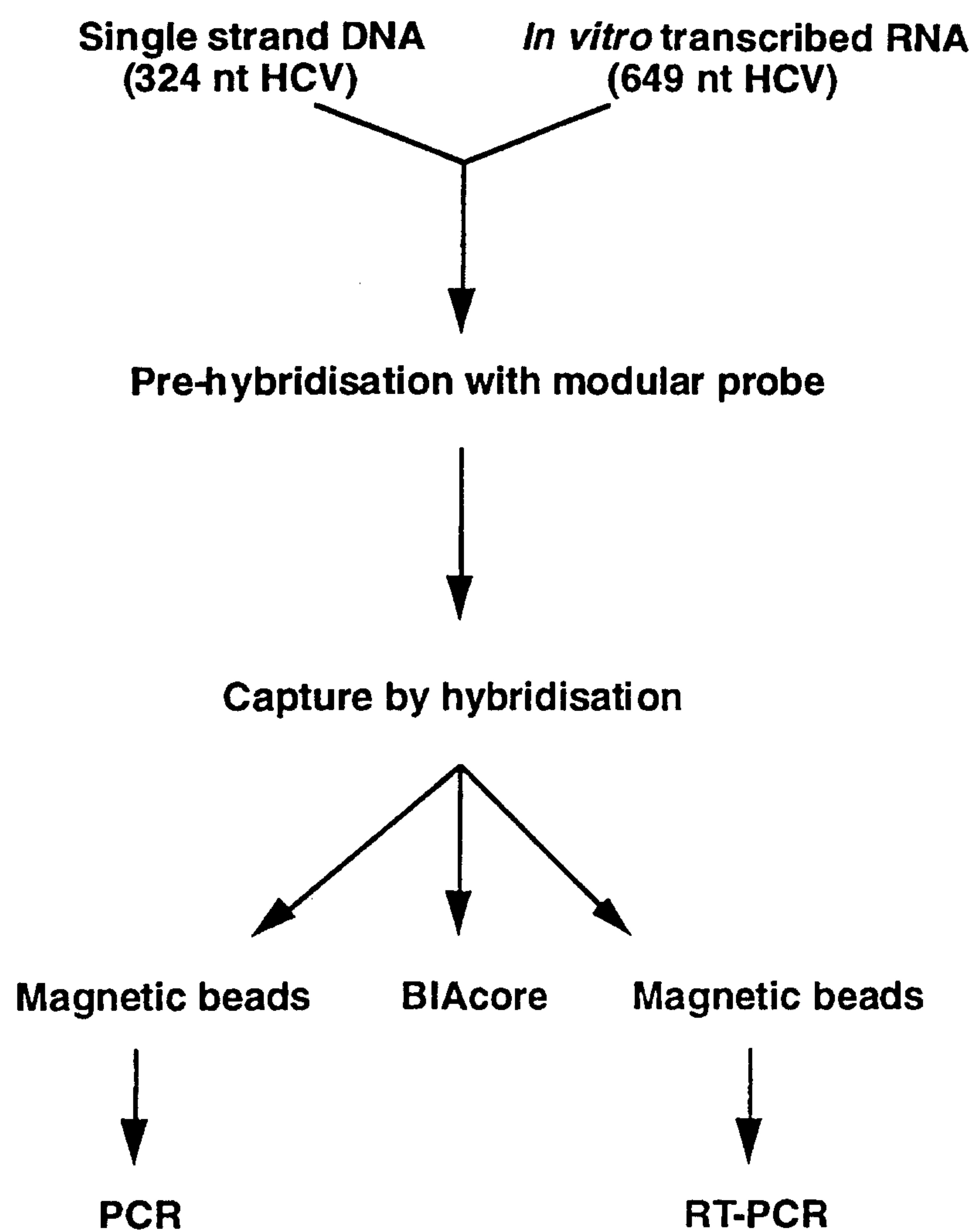


Figure 6

7 / 13

Figure 7

8/13

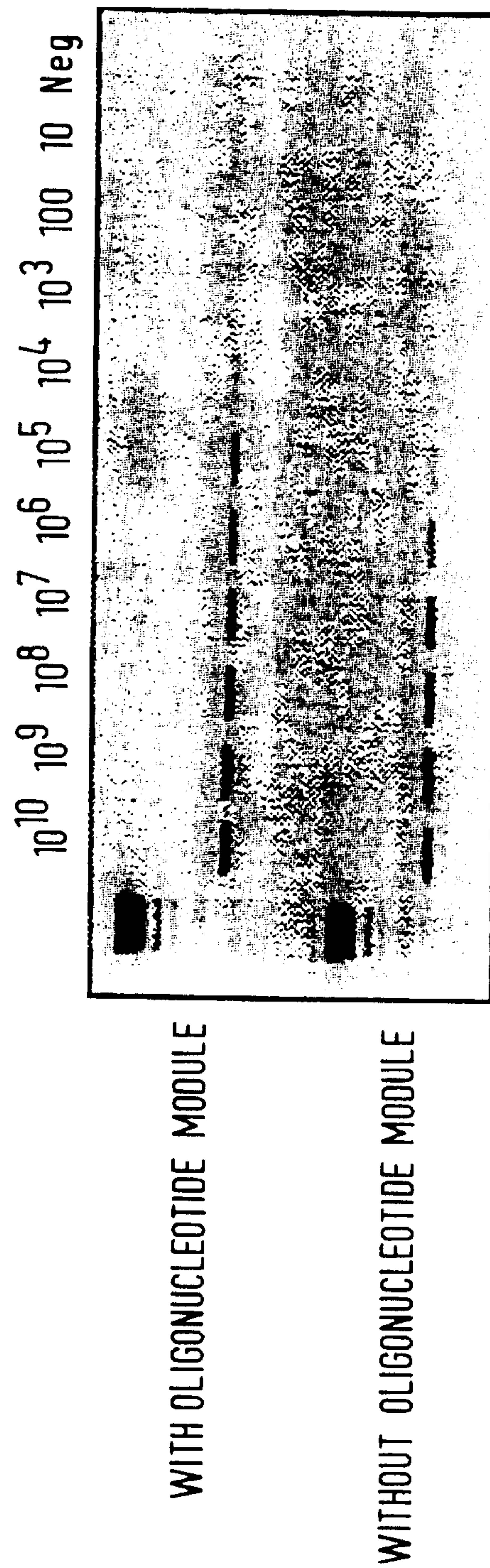


FIG. 8

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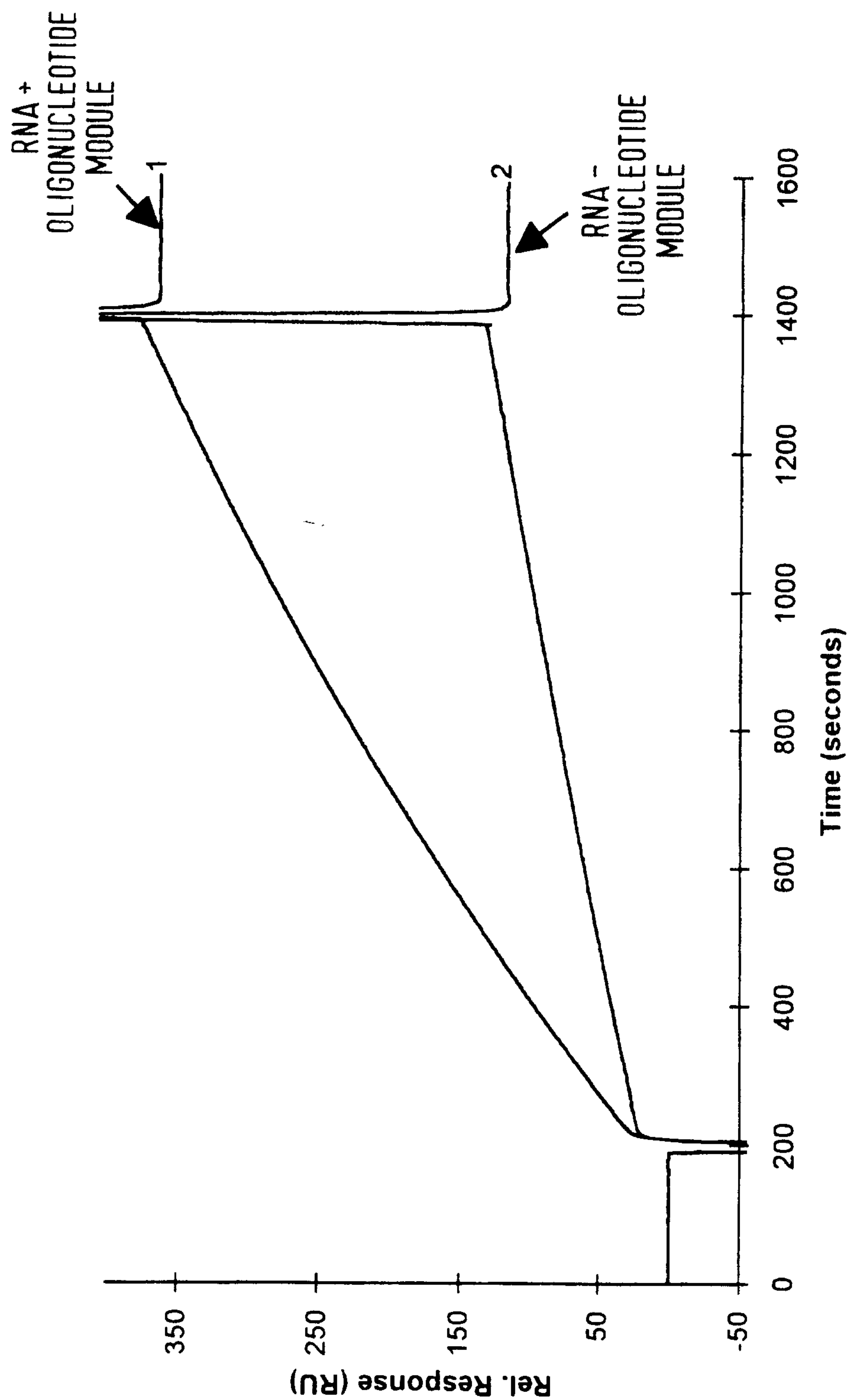


FIG. 9

10/13

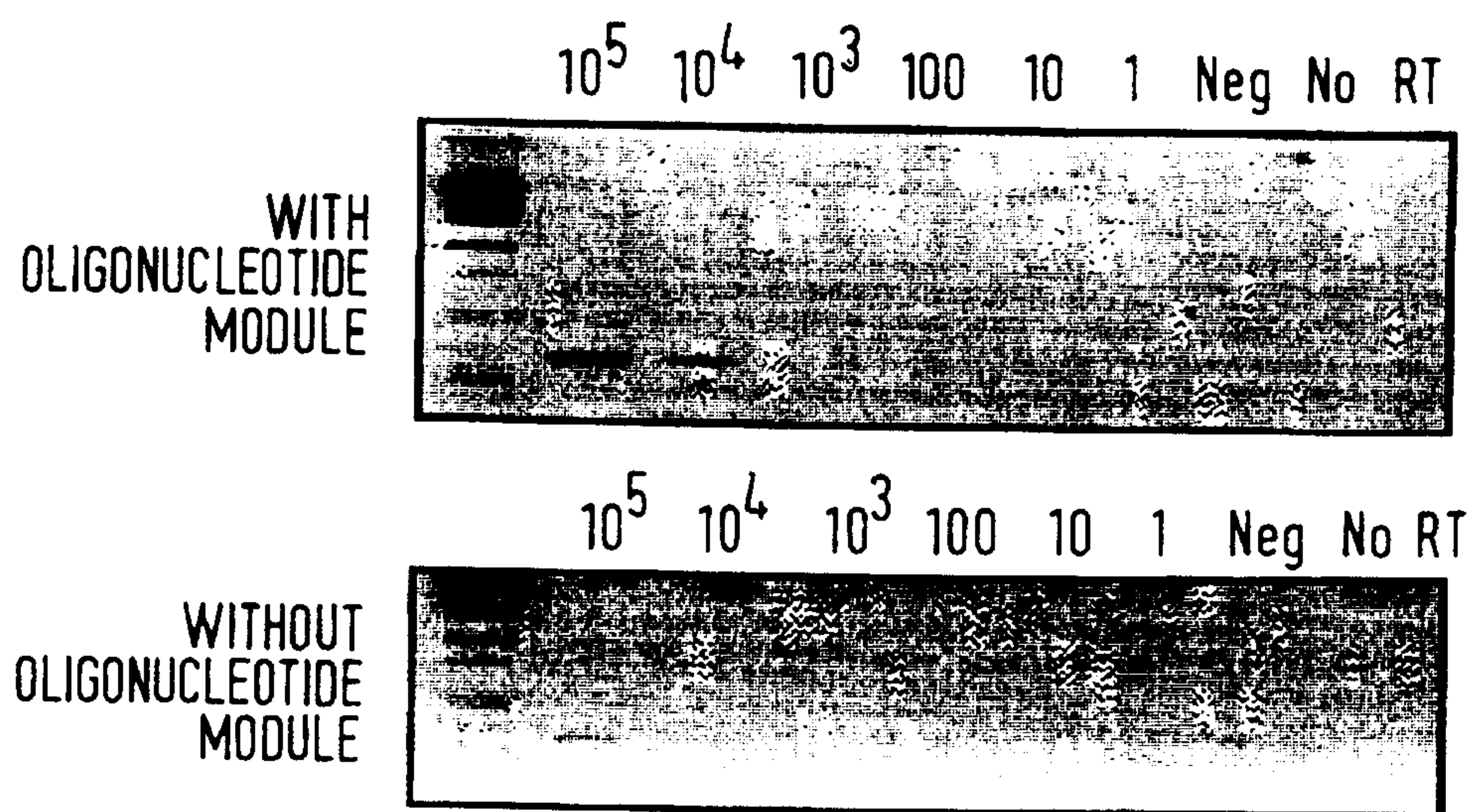


FIG. 10

11 / 13

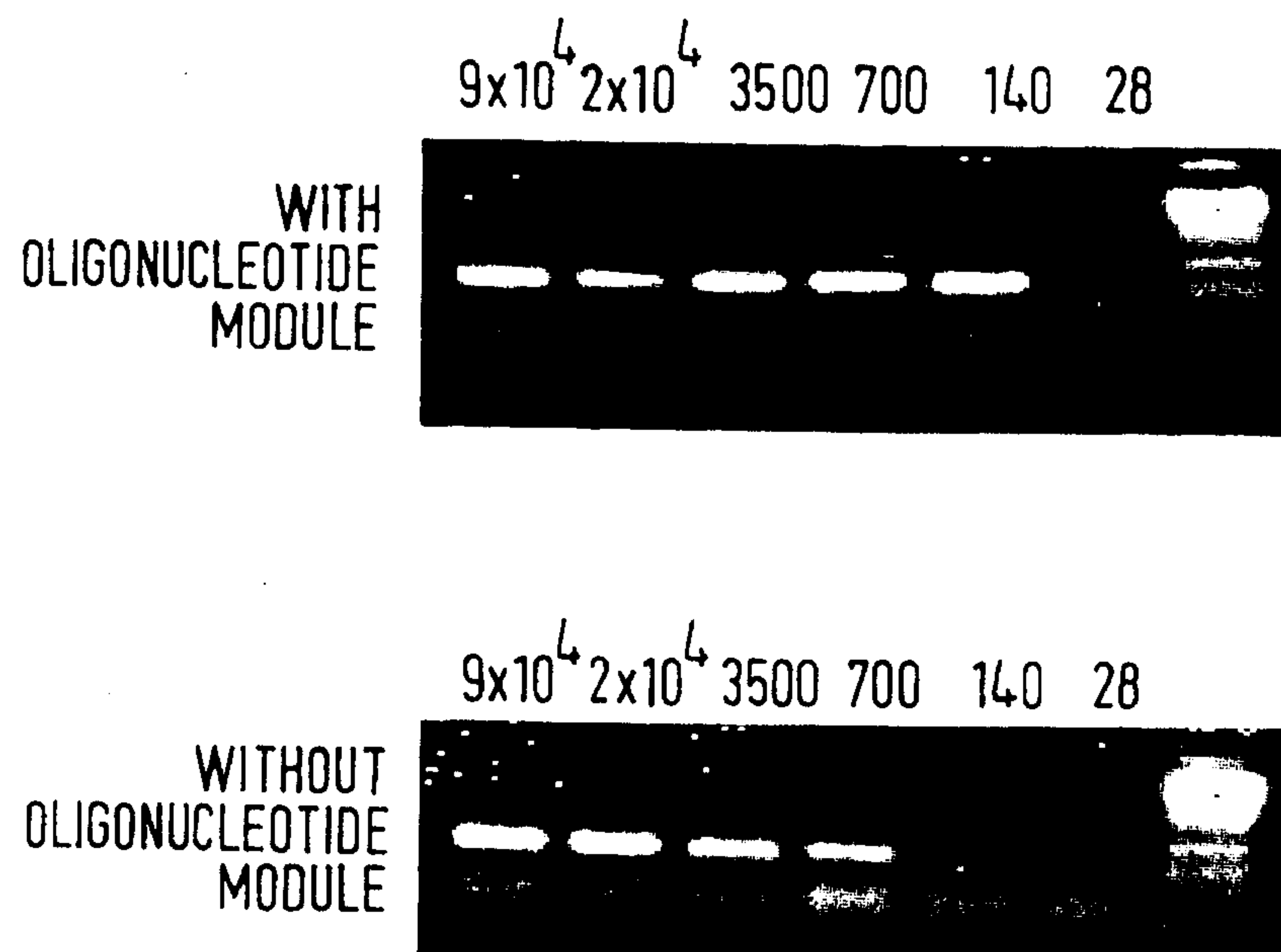


FIG. 11

12 / 13

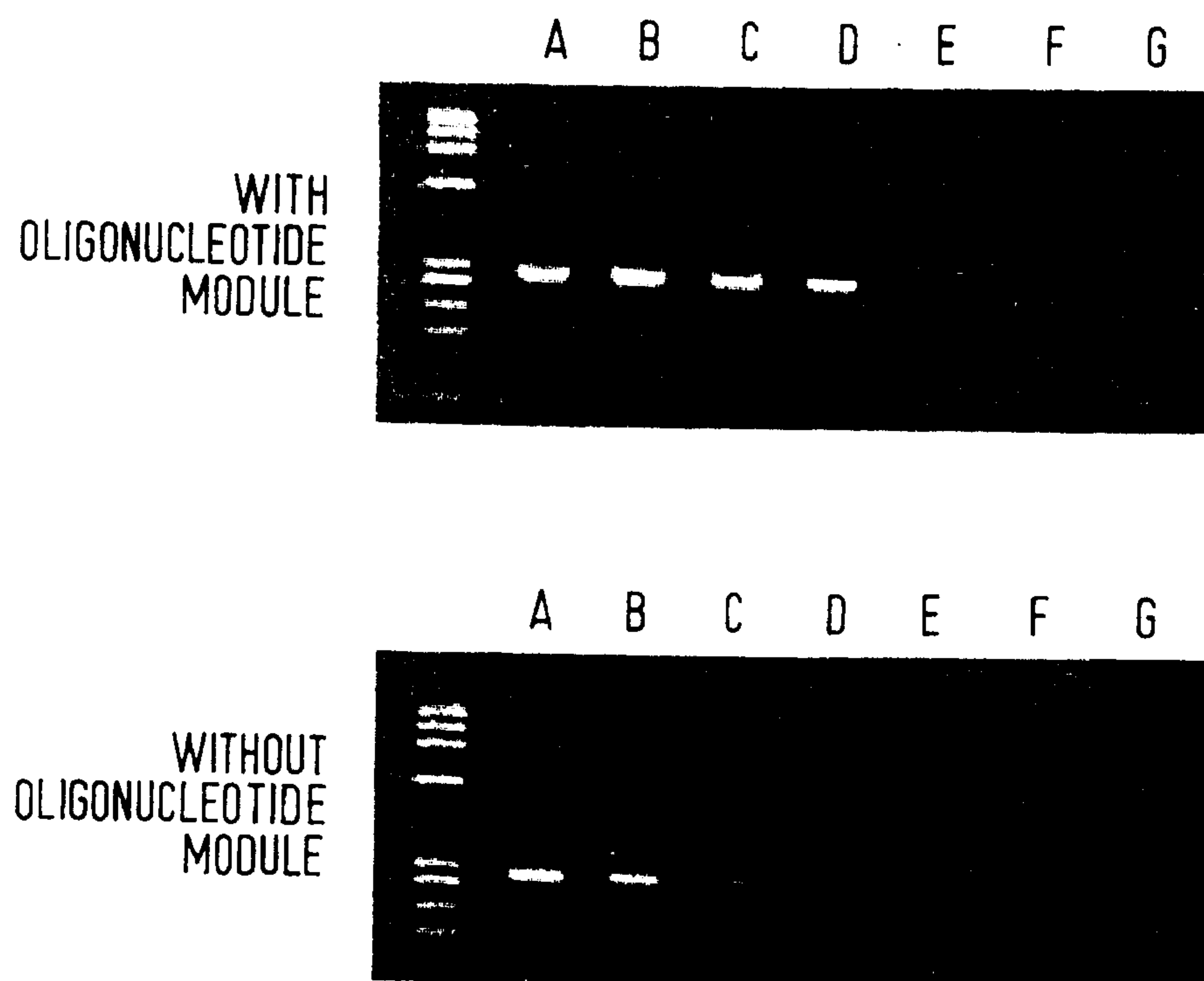
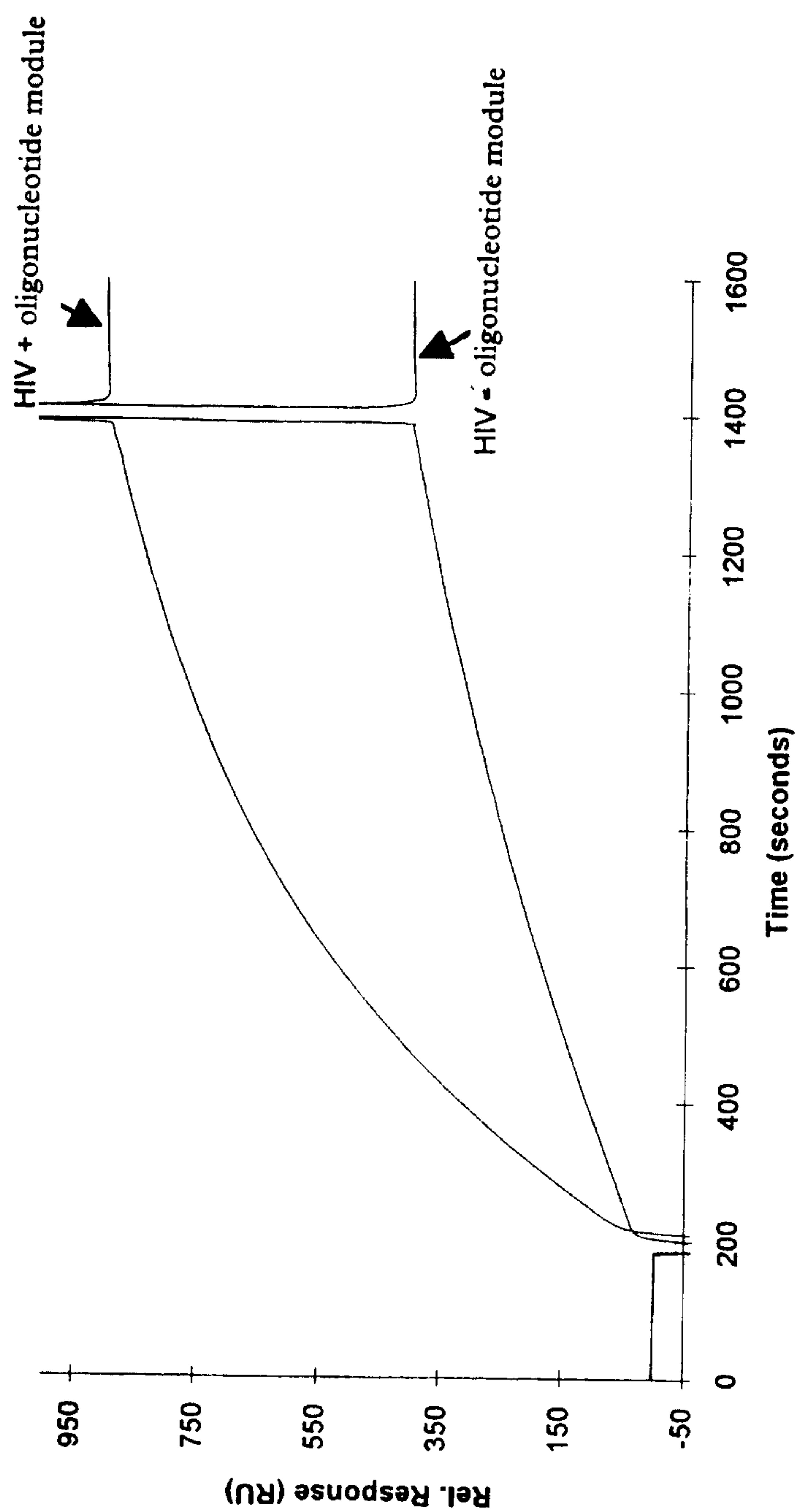


FIG. 12

13 / 13

Figure 13

