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(54) **Titre : CLONAGE ET PRODUCTION DE POLYPEPTIDES ANALOGUES DE LA FIBRONECTINE HUMAINE ET
METHODE D'UTILISATION DESDITS ANALOGUES**
(54) **Title: CLONING AND PRODUCTION OF POLYPEPTIDE ANALOGS OF HUMAN FIBRONECTIN AND METHODS
OF USING SUCH POLYPEPTIDE ANALOGS**

(57) **Abrégé/Abstract:**

This invention provides plasmids for bacterial expression of polypeptides which comprise a substantial portion of the amino acid sequence of, and which have the biological activity of, one of the domains of naturally-occurring human fibronectin, such as the cell binding domain or fibrin binding domain, comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell. In the presently preferred embodiments of the invention, the polypeptide is a 75 kD, 40 kD or 33 kD polypeptide of the cell binding domain, or a 31 kD or 20 kD polypeptide of the fibrin binding domain. The invention also provides methods for producing the polypeptides and pharmaceutical compositions comprising the polypeptides and pharmaceutically acceptable carriers. The polypeptides of this invention may be used to inhibit platelet aggregation, to inhibit thromboxane release from platelets, or to treat a subject with a cerebrovascular disorder, a cardiovascular disorder, a wound, a bacterial infection, a cancer, or to detect a fibrin thrombi. The invention further provides the polypeptides conjugated to thrombolytic agents, growth factors, serum albumin, blood factors, or polyethyleneglycol.



**CLONING AND PRODUCTION OF POLYPEPTIDE
ANALOGS OF HUMAN FIBRONECTIN
AND METHODS OF USING SUCH POLYPEPTIDE ANALOGS**

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ABSTRACT OF THE DISCLOSURE

This invention provides plasmids for bacterial expression of polypeptides which comprise a substantial portion of
10 the amino acid sequence of, and which have the biological activity of, one of the domains of naturally-occurring human fibronectin, such as the cell binding domain or fibrin binding domain, comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements
15 positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell. In the presently preferred embodiments of the invention, the polypeptide is a 75 kD, 40 kD or 33 kD polypeptide of the cell binding domain, or
20 a 31 kD or 20 kD polypeptide of the fibrin binding domain. The invention also provides methods for producing the polypeptides and pharmaceutical compositions comprising the polypeptides and pharmaceutically acceptable carriers. The polypeptides of this invention may
25 be used to inhibit platelet aggregation, to inhibit thromboxane release from platelets, or to treat a subject with a cerebrovascular disorder, a cardiovascular disorder, a wound, a bacterial infection, a cancer, or to detect a fibrin thrombi. The invention further
30 provides the polypeptides conjugated to thrombolytic agents, growth factors, serum albumin, blood factors, or polyethyleneglycol.

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**CLONING AND PRODUCTION OF POLYPEPTIDE
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BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced by Arabic numerals within parentheses. Full citations for these references may be found at the end of the specification immediately preceding the claims.

This invention relates to the cloning and production of analogs of human fibronectin and methods of using such polypeptide analogs.

Fibronectin is a glycoprotein composed of two identical subunits each of approximately 220,000 molecular weight. Two major forms of fibronectin are produced and secreted by human cells in culture and in vivo (1). The cell-associated fibronectin is relatively insoluble and participates in cell adhesion, wound healing, cell differentiation and phagocytosis. The plasma fibronectin, produced primarily in the liver, is a soluble serum protein with biological properties similar to those of cell fibronectin.

Fibronectin is considered a multifunctional modular protein since limited proteolytic cleavage produces polypeptides with distinct activities. The different functional domains of the fibronectin molecule have been
5 defined by partial proteolysis digests, and include heparin-, DNA-, fibrin-, gelatin-, and cell- binding domains (2-6).

Baralle, F.E., European Patent Publication No. 207,751,
10 published January 7, 1987, discloses the complete cDNA sequence of fibronectin. Baralle also discloses the expression of fusion proteins containing a portion of the collagen binding domain of fibronectin fused to the Escherichia coli protein β -galactosidase. Similar fusion
15 proteins are disclosed by Owens and Baralle (7). Obara, et al. (1987) disclose the expression of a portion of the cell binding domain of human fibronectin fused to Escherichia coli β -galactosidase (8). Additionally, Obara, et al. (1988) disclose the expression of portions
20 of the cell binding domain fused to β -galactosidase which have been mutagenized, i.e., site specific deletions of portions of the cell binding domain were obtained as fused proteins (9).

25 The above references disclose the expression of fusion proteins only, and do not disclose the expression of unfused proteins.

Ruoslahti and Pierschbacher, U.S. Patent No. 4,517,686,
30 issued May 21, 1985, and U.S. Patent No. 4,661,111, issued April 28, 1987, disclose a 108 amino acid polypeptide exhibiting the cell-attachment activity of fibronectin. This polypeptide may be used to promote cell

attachment to substrata, in cell cultures or medical devices. The polypeptide may also be used to coat the surface of a prosthetic device, thereby promoting cell attachment.

5 Ruoslahti and Pierschbacher, U.S. Patent No. 4,578,079, issued March 25, 1986, and U.S. Patent No. 4,614,517, issued September 30, 1986, disclose a tetrapeptide exhibiting the cell-attachment activity of fibronectin with the formula X-Arg-Gly-Asp-R-Y (RGD). The preferred
10 R is Ser, but R may also be other amino acids such as Cys or Thr. The tetrapeptide may be used to promote cell attachment to a substrate (such as a prosthetic device) by immobilizing the peptide on the substrate, to inhibit attachment to a substrate by using the peptide in
15 solubilized or suspended form, and to enhance the phagocytic activity of cells.

These tetrapeptides are also disclosed by Haverstick, et al. (19) and Ginsberg, et al. (20).

20 Pierschbacher and Ruoslahti, U.S. Patent No. 4,589,881, issued May 20, 1986, disclose a 30 amino acid polypeptide exhibiting the cell-attachment activity of fibronectin, with proposed uses as disclosed above for the 108 amino
25 acid polypeptide.

The peptides disclosed in the above patent applications, although having the activity of the cell binding domain of fibronectin, are not as active in cell binding as
30 larger fibronectin polypeptides. It is thought that the smaller peptides are not able to obtain the correct spatial conformation to fully mimic the complete fibronectin molecule. It is also believed that these polypep-

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tides are missing additional sites or regions in the cell binding domain which are important in enhancing the cell attachment activity of the fibronectin molecule.

Applicants' invention, the use of the entire cell binding domain or large fragments of the cell binding domain instead of short synthetic peptides (RGD) for in vivo experimentation has several advantages including: (i) the affinity of the plasma-derived cell binding domain (75,000 daltons) to the fibronectin receptor is about 200-fold higher compared with that of the short RGD peptides; (ii) the stability in vivo of the entire cell binding domain to proteases, especially exopeptidases, is higher than that of the short synthetic peptides; and (iii) the rate of clearance by the kidneys is much slower for the larger proteins than the synthetic short peptides, resulting in an extended half-life of the larger fragments of the cell binding domain (CBD).

Applicants' invention provides plasmids which are used to produce polypeptides. The polypeptides may be used to inhibit platelet aggregation. Drugs developed in the past to inhibit aggregation have interfered with the physiological functions of the platelet without achieving total inhibition. The polypeptides of applicants' invention overcome these difficulties by obstructing the mechanism of platelet aggregation via competition with the natural molecules which are responsible for inter-platelet interactions as well as interactions between platelets and the substratum.

Applicants' polypeptides may be used as therapeutic agents and may have a considerably longer half-life than the prostacyclins. Simultaneously, they may have

significantly shorter half-lives than aspirin or other commonly used antiplatelet aggregation drugs. Hence, they may not have the complications associated with these drugs. Even though they act during the critical stage of injured lumen blood vessel repair, applicants' polypeptides may not interfere with the indispensable coagulation-induced events necessary for wound repair. Hence, uncontrolled bleeding may not occur. This is because applicants' polypeptides may act by interfering specifically with the mechanism of adhesion and spreading of various moieties involved in aggregation, unlike existing therapies which interfere with the physiological functions of platelet aggregation. The pharmacokinetics of the therapeutics may be modified by design, thereby assuring reversibility of the process and ideal management of the coagulation and platelet aggregation process.

Several papers by Grinnell and co-workers have suggested the use of fibronectin in the healing of chronic wounds (10). Grinnell has recently reported at a scientific meeting on clinical trials in which exogenously applied, plasma-derived fibronectin enhanced wound healing. Moreover, studies by Nishida and co-workers in animal models have shown correlations between ulceration of cornea (lack of re-epithelization) and a fibronectin deficiency. This group has used exogenous plasma-derived fibronectin as a treatment for corneal ulcers in humans (11).

Wound healing therapeutics currently under development use the entire fibronectin molecule derived from pooled donor blood or autologous blood. Applicants' invention uses the individual functional domains for wound healing.

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For example, the recombinant cell binding domain may be used instead of the whole fibronectin molecule to promote wound healing. The recombinant polypeptide is more stable and may be produced in virtually unlimited amounts, free of any blood contaminants. The fibrin binding domain of fibronectin may be used as an anti-infective agent to prevent sepsis in wounds.

Humphries, et al. (12) have demonstrated that co-injection of the RGD peptide with mouse melanoma cells dramatically inhibited formation of lung metastatic colonies in mice.

Applicants' polypeptides, which have an extended half life as compared with the short synthetic RGD peptides after intravenous injection, are a more efficient inhibitor of metastasis.

Applicants' fibronectin polypeptide analogs may also be useful in detecting clotting of fibrin in leg veins, a common clinical manifestation in the elderly. Localization of a fibrin thrombus is a very complicated procedure performed in nuclear medicine departments. The assay is based on the low affinity of injected radiolabeled fibrinogen for the thrombus (21). However, the labeled fibrinogen is diluted by the large amounts of endogenous fibrinogen in the blood circulation (about 10 gr.).

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Thus, the method is not sensitive.

Applicants' invention provides for the use of the radiolabeled amino-terminal domain of fibronectin (31 kD), which has high binding affinity to fibrin, as a
5 tracer for detection of fibrin thrombi. Since the amount of fibronectin in the blood is considerably lower than that of fibrinogen, applicants' invention provides a more sensitive tracer for thrombus detection. Moreover, as
10 this domain of fibronectin is covalently cross-linked to the fibrin clot by factor XIII, considerable accumulation of the radiolabeled domain at the clot site will occur.

Finally, this invention provides novel polypeptide analogs of fibronectin which are similar in sequence to
15 domains of the human fibronectin molecule. The invention further provides uses of individual domains of fibronectin in the treatment of subjects with conditions such as cerebrovascular disorders, cardiovascular disorders, wounds, and cancer.

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SUMMARY OF THE INVENTION

This invention provides a plasmid for expression of a polypeptide which comprises a substantial portion of the amino acid sequence of one of the domains of naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

Also provided is a plasmid for expression of a polypeptide which comprises a substantial portion of the amino acid sequence of the cell binding domain of naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

The invention provides a plasmid for expression of a polypeptide which comprises a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

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In the presently preferred embodiments of the invention, the polypeptide is about a 75 kD polypeptide fragment of the cell binding domain of human fibronectin; about a 40 kD polypeptide fragment of the cell binding domain of human fibronectin; about a 33 kD polypeptide fragment of the cell binding domain of human fibronectin; about a 31 kD polypeptide fragment of the fibrin binding domain of human fibronectin; or about a 20 kD polypeptide fragment of the fibrin binding domain of human fibronectin.

10 In even more preferred embodiments, the polypeptide is a 75 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1102-1851, but deleted of amino acids 1600-1689; a 40 kD polypeptide
15 fragment of the cell binding domain of human fibronectin comprising amino acids 1380-1851, but deleted of amino acids 1600-1689; a 33 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1329-1722, but deleted of amino acids
20 1600-1689; a 31 kD polypeptide fragment of the fibrin binding domain of human fibronectin comprising amino acids 1-262; or a 20 kD polypeptide fragment of the fibrin binding domain of human fibronectin comprising amino acids 1-153 and 13 additional amino acids.

25 The plasmids are preferably expressed in suitable strains of Escherichia coli. An example of a suitable Escherichia coli strain is A4255 (F⁺) [ATCC Accession No. 67830], Escherichia coli strain A1645 [ATCC Accession No. 67829], or Escherichia coli strain A4255 [ATCC Accession
30 No. 67910].

This invention also provides a method of producing a

polypeptide which comprises a substantial portion of the amino acid sequence of one of the domains of naturally-occurring human fibronectin which comprises treating an Escherichia coli cell containing a plasmid comprising DNA encoding the polypeptide so that the DNA
5 directs expression of the polypeptide and recovering from the cell the polypeptide so expressed.

Further provided is a method of producing a polypeptide which comprises a substantial portion of the amino acid
10 sequence of the cell binding domain or the fibrin binding domain of naturally-occurring human fibronectin which comprises treating an Escherichia coli cell containing a plasmid comprising DNA encoding the polypeptide so that the DNA directs expression of the polypeptide and
15 recovering from the cell the polypeptide so expressed.

In the presently preferred embodiments of the invention, the polypeptide so produced is a 75 kD, 40 kD, or 33 kD polypeptide fragment of the cell binding domain of
20 naturally-occurring human fibronectin, or a 31 kD or 20 kD polypeptide fragment of the fibrin binding domain of naturally-occurring human fibronectin.

The invention provides a purified polypeptide substantially free of other substances of human origin which
25 comprises a substantial portion of the amino acid sequence of one of the domains of naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring
30 human fibronectin. Preferred domains are the cell binding domain and the fibrin binding domain. Preferred polypeptides are the 75 kD, 40 kD, and 33 kD polypeptides of the cell binding domain and the 31 kD and 20 kD

polypeptides of the fibrin binding domain.

The invention provides a bacterially-produced polypeptide which comprises a substantial portion of the amino acid sequence of one of the domains of naturally-occurring
5 human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin. Preferred domains are the cell binding domain and the fibrin binding domain. Preferred polypeptides are the 75 kD, 40 kD, and 33 kD polypeptides
10 of the cell binding domain and the 31 kD and 20 kD polypeptides of the fibrin binding domain.

This invention further provides a polypeptide which comprises a substantial portion of the amino acid
15 sequence of one of the domains of naturally-occurring human fibronectin which does not correspond to a product of proteolytic digestion of human fibronectin. Also provided is a polypeptide which comprises a substantial portion of the amino acid sequence of the cell binding
20 domain or the fibrin binding domain of naturally-occurring human fibronectin which does not correspond to a product of proteolytic digestion of human fibronectin.

25 Preferred polypeptides are the 75 kD, 40 kD, and 33 kD polypeptides of the cell binding domain and the 31 kD and 20 kD polypeptides of the fibrin binding domain.

The invention provides a composition comprising at least
30 two of the polypeptides described above and a suitable carrier, and a hybrid polypeptide consisting essentially of at least two such polypeptides.

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The invention further provides pharmaceutical compositions comprising an amount of the polypeptides of the subject invention effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier. Also provided are pharmaceutical compositions comprising an amount of the polypeptides of the subject invention effective to inhibit thromboxane release from platelets and a pharmaceutically acceptable carrier.

The polypeptides of this invention may be used to inhibit platelet aggregation or to inhibit thromboxane release from platelets. They may also be used to treat a subject with a cerebrovascular disorder, a cardiovascular disorder, a wound, a bacterial infection, a cancer, or to detect a tumor or thrombi (by imaging of the thrombi).

The polypeptides of this invention may be bound to thrombolytic agents, growth factors, serum albumin, blood factors, polyethyleneglycol, or superoxide dismutase.

The polypeptides of the subject invention may be used to coat a medical device, such as a catheter, so as to minimize risk of bacterial infection associated with use of such medical devices.

Further provided are methods of treating as described above wherein the polypeptide is derived by digestion of plasma fibronectin with proteases.

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BRIEF DESCRIPTION OF THE FIGURES

5 In the figures, the numbers in brackets adjacent certain of the restriction enzyme sites shown correspond to the identically numbered positions along the nucleotide sequence of human fibronectin cDNA as shown in Figure 41 (see also Figure 3 of Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1987).

10 The cDNA sequence applicants have cloned and expressed is missing the 270 bp extra domain (ED) segment which extends from nucleotides 4811 to 5080, inclusive, on the Baralle map (see Figure 41). Thus, the cDNA sequence which is said to extend from nucleotide 3317 to 5566 on the Baralle map, contains only 1980 nucleotides, because
15 it is missing the 270 nucleotides of the ED segment, namely from nucleotides 4811 to 5080 inclusive; this region is also known in the art as the ED-A region. Similarly, the protein expressed by that DNA fragment would encode from amino acid 1102 to amino acid 1851 on
20 the Baralle map but would be missing the 90 amino acids encoded by the ED region, namely amino acids 1600-1689 inclusive, and thus it would contain only 660 amino acids. This is true for all fragments described in this application which span the ED region. (The region known
25 in the art as the ED-B region is missing both in Baralle's sequence and in applicants' cDNA.)

30 The definition of the proteins expressed as 40 kD, 31 kD, 75 kD, 33 kD and 20 kD is an operational definition, based on their mobility on SDS polyacrylamide gels compared to that of markers of known molecular weight.

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Figure 1. The upper portion of Figure 1 shows various fibronectin cDNA clones which have been isolated and the alignment of such cDNA clones relative to one another and to the full length sequence of fibronectin cDNA. The lower portion of Figure 1 is a schematic
5 representation of the various domains present within the human fibronectin polypeptide.

Figure 2. This figure shows the construction of plasmid pFN100 from plasmids pBR322 and pFN919 using a
10 synthetic linker. Plasmid pFN100 encodes the 5' end of the cDNA corresponding to the fibronectin cell binding domain, which corresponds to nucleotides 3317-4090.

Figure 3. This figure shows the construction of
15 plasmid pFN101 from plasmids pBR322 and pFN919 using a synthetic linker. The plasmid pFN101 contains cDNA corresponding to nucleotides 3611-4090 of fibronectin.

Figure 4. This figure shows the construction of
20 plasmid pFN102 from plasmids pBR322 and pFN916 using a synthetic linker. An EcoRI-BglII fragment corresponding to nucleotides 4151-5566 was ligated to the synthetic linker (as shown in the figure) and the large pBR322 fragment derived from an EcoRI-Sal I digestion of pBR322.
25 Plasmid pFN102 encodes the 3' end of the cDNA corresponding to the cell binding domain.

Figure 5. This figure shows the construction of plasmid pFN103 from plasmids pFN100 (Figure 2) and pFN102
30 (Figure 4) using a synthetic linker. Plasmid pFN103 encodes the cDNA region corresponding to the cell binding domain of fibronectin from nucleotides 3317 to 5566.

Figure 6. This figure shows the construction of plasmid pFN105 from plasmid pFN103 (Figure 5) and plasmid pTV301 (Figure 7). The cDNA corresponding to the cell binding domain was removed from plasmid pFN103 and inserted into a fragment of plasmid pTV301 under the control of the λ P_L promoter and the cII ribosomal binding site. It was expected that plasmid pFN105 would direct the expression of the entire cell binding domain (75 kD). However, due to a single thymidine deletion in position 4030, a termination codon within the coding region of the cDNA was generated and plasmid pFN105 directed the expression of a polypeptide having a molecular weight of 39kD.

Figure 7. This figure shows the construction of plasmid pTV301 from plasmids pTV104(2) and p579. Plasmid pTV301 directs the expression of a human growth hormone analog under the control of the λ P_L promoter and the cII ribosomal binding site. The plasmid also contains a T₁T₂ transcription terminator downstream of the cDNA encoding human growth hormone.

Figure 8. This figure shows the construction of plasmid pFN104 from plasmids pFN101 and pFN102 using a synthetic linker. Plasmid pFN104 contains cDNA encoding the cell binding domain from nucleotides 3611 to 5566.

Figure 9. This figure shows the construction of plasmid pFN106 from plasmids pTV301 and pFN104. Plasmid pFN106 was expected to direct the expression of a 65 kD polypeptide under the control of the λ P_L promoter and the cII ribosomal binding site. The 65kD polypeptide was expected to contain the RGD sequence. However, due to a single thymidine deletion at position 4030, a termina-

tion codon within the cDNA sequence was generated and plasmid pFN106 directed the expression of a 35kD protein.

Figure 10. This figure shows the construction of plasmid pFN126-3 from plasmids pFN105 and pFN114-4. Plasmid pFN114-4 is an additional plasmid harboring FN cDNA isolated independently from the cDNA library; pFN114-4 has no deletion of the thymidine in position 4030. An SstI-BglII fragment from plasmid pFN114-4 was used to replace the SstI-BglII fragment from plasmid pFN105. The resulting plasmid pFN 117-4 directed the expression of the full length 75 kD cell binding domain protein but the plasmid does not contain the translation termination codon and terminates within the pBR322 sequence. To correct this fault, the following linker was constructed:

GATCTACTAAGGCCTA
ATGATTCCGGATTCTGA

This linker contains BglII and HindIII sites at the 5' and 3' terminals respectively, and codes for the 3' end of the 75 kD CBD including the translation termination codon. It was ligated to plasmid pFN117-4 which had been cleaved with BglII and HindIII. The resulting plasmid pFN 126-3 directed the expression of the full length 75 kD cell binding domain protein, with the authentic C-terminus.

Figure 11. This figure shows the construction of plasmid pFN118 (alternatively designated pFN 118-2) from plasmids pTV301 and pFN102 using a synthetic linker. Plasmid pFN118 was designed to express a 40 kD cell binding domain protein. However, due to a mistake in the

synthesis of the linker the protein was not terminated within the linker, but rather was only terminated in the sequences derived from the vector pBR322. Thus, the protein obtained was a fused protein.

5 Figure 12. This figure shows the construction of plasmid pFN132-5 from plasmid pFN118 and plasmid pTV301 using a synthetic linker. The synthetic linker was used to replace a portion of the defective synthetic linker in plasmid pFN118. The resulting plasmid pFN132-5
10 directed the expression of a 40kD cell binding domain protein.

Figure 13. This figure shows the collagen and DNA synthesis in applicants' wound healing model. The
15 experiments were carried out as described in Examples 10 and 22, with 50 μ g of the 40 kD protein per sponge. Bovine serum albumin (BSA) at 100 μ g per sponge and saline were used as negative controls.

20 Figure 14. This figure shows the pharmacokinetics of the 40kD recombinant CBD protein.

Figure 15. This figure shows the nucleotide sequence of a BamHI-EcoRI synthetic fragment used in the subcloning of the cell binding domain.
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Figure 16. This figure shows the construction of plasmid p579. The rRNA operon T_1T_2 transcription termination fragment was isolated from plasmid pPS1 (deposited with the ATCC under Accession No. 39807) which had
30 been digested with HindIII. The T_1T_2 fragment was inserted into the unique HindIII site of pRO211 (Figure 17) which had been digested with HindIII. The resulting

expression vector, p579, contains the λ P_L promoter and the C_{II} ribosomal binding site, followed by the T₁T₂ transcription termination sequences.

Figure 17. This figure shows the construction of plasmids pRO211 and pRO12. The plasmid pJH200 (deposited with the ATCC under Accession No. 39783) was partially digested with NdeI, treated with DNA polymerase I (Klenow) to fill in the ends and the resulting ends were religated to form the expression vector pRO211. The expression vector pRO211 was digested with NdeI and HindIII, the large fragment isolated and ligated to an NdeI-HindIII bovine growth hormone (bGH) fragment isolated from pAL500 (deposited with the ATCC under Accession No. 39782) to give pRO12. (The NdeI-HindIII fragment was produced from pAL500 by digesting it with EcoRI and ligating to the ends of the digestion product synthetic linkers with the sequence:

TATGGATC
ACCTAGTTAA

The ligation mixture was digested with NdeI and HindIII and the resulting NdeI-HindIII bGH fragment isolated.)

Figure 18. This figure shows various fibronectin plasmids which contain the cell binding domain, and the alignment of the plasmids to one another and to the full length sequence of the cell binding domain. (The alignment of the cell binding domain relative to the fibronectin cDNA is shown in Figure 1.) The molecular weight of the protein expressed by each plasmid, as determined by electrophoretic mobility on SDS-polyacrylamide gels, is indicated in the right hand column in

kiloDaltons (kD); each protein is designated according to its molecular weight, e.g., the 75 kD protein.

The numbers in brackets correspond to the identically numbered positions along the nucleotide sequence of human fibronectin cDNA shown in Figure 41 (see also Figure 3 of Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1987). The extra domain (ED) is missing; this corresponds to nucleotides 4811 to 5080 inclusive (270 nucleotides) encoding amino acids 1600 to 1689 inclusive (90 amino acids). (This ED region is also known in the art as the ED-A region; the ED-B region is missing both in Baralle's sequence and in applicants' cDNA.)

15 CBD I = Cell Binding Domain I (includes the RGD sequence)

CBD II = Cell Binding Domain II [as disclosed by Obara et al., Cell 53: 649-657 (1988)]

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Figure 19. This figure summarizes information about the plasmids encoding various fragments of the cell binding domain and the proteins expressed by them.

25 Figure 20. This figure shows the construction of plasmid pFN128-4 by ligation of synthetic linker A (Figure 26) to the large fragment produced by EcoRV and BglII digestion of plasmid pFN117-4 (Figure 10). Plasmid pFN128-4 contains cDNA encoding the cell binding domain from nucleotides 3317 to 5179; the 3' terminus is formed by linker A. The plasmid expresses a 65 kD cell binding domain protein under the control of the λP_L promoter and the cII ribosomal binding site. Note that this plasmid

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could have been identically constructed from plasmid pFN126-3 (ATCC Accession No. 67829), which differs from plasmid pFN117-4 only at the 3' terminus of the cDNA encoding the cell binding domain.

5 Figure 21. This figure shows the construction of plasmid pFN130-11 by ligation of synthetic linker B (Figure 26) to the large fragment obtained by digestion of plasmid pFN117-4 by BamHI and HindIII. Plasmid pFN130-11 contains cDNA encoding the cell binding domain
10 from nucleotides 3317 to 4090; the 3' terminus was formed by linker B. This plasmid expresses a 28 kD cell binding domain protein under the control of the λ P_L promoter and the cII ribosomal binding site. Note that this plasmid could have been identically constructed from
15 plasmid pFN126-3 (ATCC Accession No. 67829), which differs from plasmid pFN117-4 only at the 3' terminus of the cDNA encoding the cell binding domain.

Figure 22. This figure shows the construction of
20 plasmid pFN135-12 by tri-partite ligation of synthetic linker C (Figure 26), synthetic linker D (Figure 26) and the large fragment obtained by digestion of plasmid pFN117-4 by NdeI and BamHI. Plasmid pFN135-12 contains cDNA encoding the cell binding domain from nucleotides
25 3998 to approximately 5566; the 5' terminus was formed by linkers C and D. This plasmid expresses a 45 kD cell binding domain protein under the control of the λ P_L promoter and the cII ribosomal binding site. Note that there is the same error in the C-terminus of this protein
30 as in the 75 kD protein expressed by the parent plasmid pFN117-4 (see Description to Figure 10); i.e., there is no translation termination codon at the end of the fibro-

nectin cDNA, and thus translation terminates in the pBR322 coding sequence.

5 Figure 23. This figure shows the construction of plasmid pFN137-2 by ligation of synthetic linker A (Figure 26) to the large fragment produced by EcoRV and BglII digestion of plasmid pFN135-12 (Figure 22). Plasmid pFN137-2 contains cDNA encoding the cell binding domain from nucleotides 3998 to 5179; the 3' terminus was formed by linker A. This plasmid expresses a 33 kD cell
10 binding domain protein under the control of the λ P_L promoter and the cII ribosomal binding site. Plasmid pFN137-2 has been deposited with the ATCC under Accession No. 67910.

15 Figure 24. This figure shows the construction of plasmid pFN143-1 by tri-partite ligation of synthetic linker E (Figure 26), the large fragment obtained by digestion of plasmid pFN137-2 by NdeI and BamHI, and the small fragment obtained by digestion of plasmid pFN126-3
20 by SstI and BamHI. Plasmid pFN143-1 contains cDNA encoding the cell binding domain from nucleotides 3602 to 5179; the 5' terminus was formed by linker E. This plasmid expresses a 55 kD cell binding domain protein under the control of the λ P_L promoter and the cII
25 ribosomal binding site.

Figure 25. This figure shows the construction of plasmid pFN134-9 by triple ligation of synthetic linker A (Figure 26), the large fragment derived by digestion
30 of plasmid p578 (see below) by NdeI and BglII, and the small fragment derived by digestion of plasmid pFN118 (Figure 11) by NdeI and EcoRV. Plasmid pFN134-9 contains cDNA encoding the cell binding domain from nucleotides

4151 to 5179; the 3' terminus was formed by linker A. Downstream of the 3' terminus are the T₁T₂ transcription termination sequences.

5 The plasmid expresses a 28 kD cell binding domain protein under the control of the λ P_L promoter and the cII ribosomal binding site.

Plasmid p578 is another isolate from the same ligation that produced p579 (see Figure 16). Plasmids p578 and
10 p579 are understood to be identical.

Note that plasmid pFN134-9 could have been constructed in a similar manner from plasmid pFN132-5 (ATCC Accession No. 67830) which differs from plasmid pFN118 only at the
15 3' terminus of the cDNA encoding the cell binding domain.

Figure 26. Synthetic linkers were used in the construction of the subject plasmids. This figure shows the base sequence of synthetic oligonucleotides A, B, C,
20 D and E used as linkers in the construction of plasmids described in the preceding figures.

Figure 27. This figure shows the pharmacokinetics of the radioactively-labeled 33 kD protein when injected
25 intravenously into rats.

Figure 28. This figure shows the dose-response effect of the 33 kD and 40 kD proteins and also of the plasmatic 75 kD protein (P-75 kD) and the synthetic pentapeptide
30 GRGDS (Sigma) on the in vitro human platelet aggregation assay system described in Example 7. (The plasmatic 75 kD protein was purified from a tryptic digest of fibronectin by an adaptation of the method described by

Hayashi, M. and Yamada, K.M. (28).) The proteins (and GRGDS) were added in the amounts shown in PBS solution to separate reaction tubes containing 125 μ l of platelet-rich plasma (PRP) (from donor T). The final reaction volume was made to 250 μ l by the addition of PBS after equilibration. Platelet aggregation was induced by addition of ADP solution to a final concentration of 10 μ M ADP. The transmittance of each reaction tube was measured (during 3-minute reaction time) and inhibition of platelet aggregation, calculated as a percent maximum transmittance, was plotted against protein concentration (μ M). (In aggregation experiments fresh blood was used and, because of variation in platelet behavior depending on the source, the donor, T or R, has been indicated.)

Figure 29. This figure shows the dose-response effect of the 33 kD and the 40 kD proteins on platelet aggregation in whole blood, using the in vitro platelet aggregation assay system described in Example 7, modified as follows: the reaction tube mixture contained 500 μ l whole blood (from R) plus 500 μ l PBS or protein solution in PBS. The experiments were performed in the presence of 0 (control), 0.65 μ M and 2.6 μ M 33 kD protein and in the presence of 0 (control), 1.5 μ M and 7.5 μ M 40 kD protein. The solutions were equilibrated to 37°C for 3 minutes, and then platelet aggregation was induced by addition of ADP solution to a final concentration of 10 μ M ADP.

For whole blood solutions, impedance was monitored instead of transmittance. The impedance method detects aggregation by passing a very small electric current between two electrodes immersed in a sample of blood, and measuring the electrical impedance between the electrodes

(22). The impedance results in the above experiments were converted to percent inhibition (calculated as percentage of maximum impedance in control tubes), and plotted against the concentration (μM) of the 33 kD and 40 kD proteins.

5 The 40 kD protein sample had been reactivated by urea treatment as described in Example 20.

10 Figure 30. This figure shows the effect of the 33 kD and 40 kD proteins in the in vitro platelet aggregation system described in Example 7. Platelet-rich plasma (from R) was used and the 33 kD and 40 kD proteins were added to the reaction tube mixture in the concentrations indicated. Two different preparations of 40 kD protein were tested: the 40 kD sample designated "TSK-1-
15 Urea-PBS" was reactivated by urea treatment as described in Example 20, after Fractogel filtration (as described in Example 15); the other 40 kD preparation was not reactivated. Calculation of inhibition of aggregation was performed by comparing the aggregation after 0.5 min.
20 reaction time.

25 Figure 31. The effect of protein concentration on the binding to platelets of the 33 kD and 40 kD proteins and human plasmatic fibronectin was examined using the method described in Example 19. The reaction mixture contained 5×10^8 washed platelets and thrombin. In each case the amount of labeled protein was as indicated in the figure.

30 Figure 32. The effect of the synthetic pentapeptide GRGDS (Sigma) on the binding to washed platelets of human plasma fibronectin (h-FN) and the 40 kD and 33 kD proteins was examined using the method described in

35 ,

Example 19 and using thrombin as stimulant. Experiments were performed using $0.1 \mu\text{M}$ ^{125}I -labeled 40 kD, 33 kD and plasmatic FN alone and also in the presence of a 10-fold excess of cold (unlabeled) homologous protein (40 kD and 33 kD respectively) and in the presence of $50 \mu\text{M}$ GRGDS pentapeptide. The figures were normalized by considering the binding by each labeled protein alone as 100%.

Figure 33. The effect of the presence or absence of thrombin on the binding of the 40 kD and 33 kD proteins to washed platelets was examined using the method described in Example 19. The platelet concentration was 5×10^8 platelets/ml.

- A. Each reaction mixture contained 100 nM ^{125}I -40 kD in the presence or absence of thrombin and in the presence or absence of additional $1 \mu\text{M}$ unlabeled 40 kD, as indicated.
- B. Each reaction mixture contained 100 nM ^{125}I -33 kD in the presence or absence of thrombin and in the presence or absence of additional $1 \mu\text{M}$ unlabeled 33 kD, as indicated.

The percentage binding indicated is the percentage of input radioactive label which was bound.

Figure 34. The effect of the 40 kD and 33 kD proteins, plasmatic 75 kD (p-75 kD) and GRGDS on the binding of fibrinogen to washed platelets was studied. The method was as described in Example 19, except as follows. The reaction mixture contained 5×10^8 platelets and the experiment was performed both in the presence and absence of thrombin. After pre-incubation for 10 minutes at

25°C, 4 units per ml of hirudin (Bio-Makor, Israel) was added and pre-incubation continued for 5 additional minutes at 25°C. To the reaction mixture 100 μ M ^{125} I-fi-
brinogen was added. The control samples contained no further additions, and the test samples contained 5 μ M
5 40 kD, 5 μ M 33 kD, 5 μ M plasmatin 75 kD, or 50 μ M GRGDS pentapeptide, added at the same time as the ^{125} I-fi-
brinogen, all as indicated in the figure. Incubation continued for an additional 20 minutes before termination of the reaction and measurement of radioactivity. The
10 percentage binding indicated is the percentage of input radioactive label which was bound.

Figure 35. The dose response of the binding of the 40 kD and 33 kD proteins to washed platelets in the
15 presence and absence of thrombin was determined using the method described in Example 19. The reaction mixture contained 5×10^8 platelets.

A. ^{125} I-40 kD was added in the amounts indicated to the
20 reaction mixture.

B. ^{125} I-33 kD was added in the amounts indicated to the reaction mixture.

25 Figure 36. A time course of the binding of the 33 kD recombinant protein to washed platelets was obtained using the method described in Example 19; the reaction mixture contained 100 nM ^{125} I-33 kD and the incubation times were 5, 15, 25 and 35 minutes. The experiment was
30 performed both in the presence and absence of thrombin as indicated.

Figure 37. The binding to washed platelets of FN, the 40 kD protein, and the 33 kD protein was studied using the method described in Example 19. The reaction mixture contained 5×10^8 platelets and 100 nM of either ^{125}I -FN or ^{125}I -40 kD or ^{125}I -33 kD respectively. Incubation was carried out in the presence or absence of thrombin as indicated.

A. Incubation time = 30 minutes.

10 B. Incubation time = 10 minutes.

Figure 38. The cell binding activity of the 40 kD protein is compared to that of plasmatic fibronectin (FN).

15

A. The cell binding activity of the 40 kD protein before and after urea reactivation, 40 kD and 40 kD(U), and of plasmatic fibronectin, FN, was measured by a cell attachment assay system (23) (see Example 16), using a BALB/C 3T3 fibroblast cell line (ATCC Accession No. CCL 163). Urea reactivation was carried out as described in Example 20.

20

B. The cell binding activity of the 40 kD protein and of plasmatic fibronectin (FN) was measured by a cell attachment assay system using either the 3T3 fibroblast cell line described above or an NRK-52E cell line (ATCC Accession No. CRL 1571). The assay technique was as described in A above.

25

30

Figure 39. The cell binding activity of the 40 kD and 33 kD recombinant proteins and plasmatic fibronectin (FN) was measured by a cell attachment assay system as

35.

described in Figure 38. The BHK cells used are described by Yamada. The 1/2 maximum attachment for fibronectin is obtained at 0.8 $\mu\text{g/ml}$ and for the 40 kD protein is obtained at 2.4 $\mu\text{g/ml}$.

5 Figure 40. This figure shows the effect of the 40 kD protein on collagen and DNA synthesis in a sponge wound healing model in rats. The experiments were carried out as described in Example 22. Plasma fibronectin (FN) and epidermal growth factor (EGF, Biomedical Technologies Inc.) were used as positive controls at the level of 100 μg per sponge and 8 μg per sponge, respectively.

10 Figure 41. This figure shows the nucleotide sequence of human fibronectin cDNA.

15 Figure 42. Seven pairs of chemically synthesized oligomers were prepared. The synthetic oligomers code for the first 153 N-terminal amino acids of human FN. This figure shows the sequence of these 7 pairs of synthetic oligomers.

20 Figure 43. The DNA fragment coding for amino acids 1 to 153 of N-terminal domain of human FN was assembled from the 7 pairs of chemically synthesized oligomers shown in Figure 42 as follows:

25 Oligomers 3/4, 5/6, 7/8 and 9/10, each pair in a separate tube, were annealed and then phosphorylated at the 5' end using T4 polynucleotide kinase enzyme.

30 In the second step, pairs 3/4 and 5/6 were ligated to each other using T4 DNA ligase. Similarly, reaction pairs 7/8 and 9/10 were ligated to each other. After each step of ligation an aliquot of the ligation mixture

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was analyzed on gel to determine the size of the newly formed fragments and the efficiency of ligation.

In the third step, the two above mentioned ligation mixtures were mixed together and pair 6, oligomers 11/12 which had been annealed and phosphorylated previously in a separate tube were added to the mixture. A 326 base pair DNA fragment obtained from the above ligation mixture was isolated from an agarose gel and purified.

The purified synthetic 326 fragment was added to two additional pairs of synthetic linkers: Pair 1, oligomers 1/2 and Pair 7 oligomers 13/14. In Pair 1 only oligomer 2 was phosphorylated at the 5' end and in Pair 7 only oligomer 13 was phosphorylated at the 5' end.

After ligation with T4 DNA ligase the mixture without any further isolation was added to pBR322 vector DNA digested with EcoRI and BamHI endonucleases.

The plasmid obtained, designated pFN 932-18 contained the entire synthetic EcoRI (5' end) - BamHI (3' end) restriction fragment coding for N-terminal 153 amino acids of human FN, in a pBR322 vector.

Figure 44. Expression of the N-terminal 153 amino acid sequence of FN.

Plasmid pFN 932-18 was digested with NdeI and BamHI endonucleases. The NdeI-BamHI DNA fragment coding for FN (first 153 amino acids + additional N-terminal methionine) was isolated and ligated into the large fragment obtained by digestion of plasmid pTV301 with NdeI and Bgl II endonucleases. (Plasmid pTV301 (Figure 7) expresses

human growth hormone, hGH, under the control of lambda P_L promoter and the cII RBS).

The plasmid obtained was designated pFN949-2.

Figure 45. Insertion of termination codon TAA at the
3' end of the N-terminal domain of FN (at
amino acid 262)

A synthetic oligonucleotide containing a TAA termination codon and a BglIII site having the following sequence:

CTGTTTAAGCA
GACAAATTCGTCTAG

was ligated to the 3' end (PvuII site) of an EcoRI-PvuII
FN fragment isolated from cDNA clone plasmid p931-5 (see
Figure 1) digested with EcoRI and PvuII. The ligation
was carried out in the presence of DNA vector plasmid
pBR322 digested with EcoRI and BamHI (large fragment).
The plasmid obtained was designated pFN935-12.

Figure 46. Subcloning of the carboxy-terminal region of FBD in λ P_L expression vector.

Plasmid pFN 935-12 was digested with EcoRI and HincII. The EcoRI-HincII fragment coding for FN was isolated and ligated to DNA, the large fragment obtained by digestion of plasmid pTV194-80 with EcoRI and SmaI. (Plasmid pTV194-80 expresses human ApoE under the control of the λ P_L promoter and β -lactamase promoter and RBS). The plasmid obtained was designated pFN 946-12. This plasmid is deleted of the PBLA sequences and therefore does not express the carboxy domain of FBD.

The construction of pTV194-80 from plasmid p579 (Figure 16) is shown in Figures 70 and 71.

Figure 47. Construction of the DNA fragment coding for the FBD from nucleotide No. 14 to nucleotide No. 599

Three pairs of chemically synthesized oligomers with the following DNA sequences:

Pair 1

#15 5'-TGAGAAGTGTTTTGATCATGCTGCTGGGACTTCCTATGTGG-3'

#16 3'-CTTCACAAACTAGTACGACGACCCTGAAGGATACACCAGCCT-5'

Pair 2

#17 5'-TCGGAGAAACGTGGGAGAAGCCCTACCAAGGCTGGATGATGGTAG-3'

#18 3'-CTTTGCACCCTCTTCGGGATGGTTCCGACCTACTACCATCTAACA-5'

Pair 3

#19 5'-ATTGTACTTGCCTGGGAGAAGGCAGCGGACGCATCACTTGCACCTT-3'

#20 3'-TGAACGGACCCTCTTCCGTGCGCTAGTGAACGTGAAGATC-5'

were used to carry out this construction.

Oligomers 15/16 and 17/18 were annealed and phosphorylated at the 5' end each in a separate tube and then mixed together for ligation using T4 DNA ligase. After 3 hours of ligation, oligomers 19/20 (previously annealed and kinased at their 5' ends) were added for an additional 3 hours ligation at room temperature.

The synthetic DNA fragment obtained was used for further ligation with an EcoRI-DdeI FN coding sequence obtained from plasmid pFN 932-18 digested with EcoRI and DdeI. The ligation was carried out in the presence of plasmid pUC19 digested with EcoRI and XbaI (large fragment).

The plasmid obtained was designated pFN948-4.

15 Figure 48. Construction of the entire FBD region

Plasmid pFN948-4 was digested with EcoRI and XbaI. The EcoRI-XbaI fragment coding for the N-terminal region of FBD was isolated and ligated to the carboxy terminal region of FBD by digestion of plasmid pFN946-12 with EcoRI and XbaI (using the large fragment). The plasmid obtained was designated pFN 957.

25 Figure 49. Expression of the entire FBD protein under the control of the λ P_L promoter and λ cII RBS

Plasmid pFN 957 was digested with NdeI and HindIII. The NdeI-HindIII fragment coding for the FBD was isolated and ligated into the isolated vector fragment of plasmid pTV301 digested with NdeI and HindIII in the presence of isolated purified HindIII-HindIII T₁T₂ coding DNA fragment. The plasmid obtained was designated pFN962-3.

Figure 50. Expression of the entire FBD protein under
 λ P_L promoter and PBLA ribosomal binding
site

Plasmid pBLA11 (ATCC No. 39788) was digested with EcoRI
5 and AluI. The EcoRI-AluI fragment coding for the β -
lactamase promoter and β -lactamase RBS was isolated and
ligated into plasmid pFN962-3 (Figure 49) digested with
NdeI, then treated with Klenow enzyme in the presence of
all four dNTPs to fill in the NdeI site and digested with
10 EcoRI (using the large fragment). The plasmid obtained
was designated pFN 975-25.

Figure 51. Refolding/reoxidation and purification of
r31 kD as followed by SDS-PAGE under
15 reducing and non-reducing conditions

The gel (12% acrylamide) under reducing conditions (with
 β -mercaptoethanol (ME)) monitors the process of purifica-
tion, whereas the non-reducing conditions (without ME)
20 are indicative of the refolding/reoxidation, leading to
faster moving and less diffuse bands. Note (in the
absence of ME) that the band of 'After Phenyl-S' is much
sharper than that of 'Refolded', indicating that reoxida-
tion continues even during the purification.

25 **Refolded (GSH/GSSG):** r31kD which has been refolded/reoxi-
dized - after having been extracted from the crude pellet
in the presence of GSH/GSSG 3 mM/0.3 mM at pH 8.0;
Phenyl-S; Phenyl-Sepharose; Q-S; Q-Sepharose; p31 kD;
30 plasma-derived 31 kD (obtained by tryptic digestion);
molecular weight markers: Low Molecular Weight protein
calibration kit (Pharmacia Fine Chemicals), containing
markers whose molecular weights are 94 kD, 67 kD, 43 kD,
30 kD, 20.1 kD and 14.4 kD.

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Figure 52. Purification of GSH/GSSG-refolded r31 kD
by Phenyl-Sepharose chromatography

A suspension of refolded/reoxidized and "scrambled" 31 kD, as well as insoluble contaminants, which has been
5 extracted from 10 grams of pellet, was subjected to centrifugation at 13,000 rpm (see Section 3.1 in Example 24). The supernatant (1,280 ml) was brought to 0.2 M in ammonium sulfate (AS) and loaded onto a 45 ml column of phenyl-Sepharose previously equilibrated with Buffer A
10 at pH 8.5, containing also 0.2 M AS. The column was washed with 150 ml of the same solution, followed by 150 ml of Buffer A, 50 ml of water and 50 ml of 6 M GuCl. The purified r31kD appeared in the Buffer A fraction and at this stage it was more than 85% pure. Absorbance was
15 measured at 280 nm.

Figure 53. Concentration and purification of r31 kD
by Heparin-Sepharose chromatography

20 Approximately 1/2 of the Buffer A peak from the phenyl-Sepharose step (see Figure 52) was concentrated and purified on a 10 ml Heparin-Sepharose column, from which it was eluted by a solution of 0.5 M NaCl in Buffer A. At this stage the r31 kD is more than 90% pure. Absor-
25 bance was measured at 280 nm.

Figure 54. Purification of r31 kD by Q-Sepharose
chromatography

30 Concentrated r31 kD (Figure 53), which had been dialyzed against Buffer A, pH 8.5, was loaded on a 40 ml column of Q-Sepharose, which had previously been equilibrated with the same buffer. The purified r31 kD, which eluted in the flow-through and wash fractions, was concentrated
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by lyophilization. The column was washed free from the contaminant proteins by a step of 1 M NaCl. The purified r31 kD is at this stage more than 95% pure. Absorbance was measured at 280 nm.

Figure 55. Analytical FPLC-gel permeation of r31 kD on a Superose 12 column

5

A mixture of plasma derived and recombinant 31kD (100 μ l at 0.8 mg/ml) in running buffer (20 mM Tris.HCl - 150 mM NaCl. pH 7.8) was applied onto the Superose 12 column (HR 10/30), pre-equilibrated in running buffer, and eluted from it in the same buffer. Flow rate - 0.4 ml/minute; chart speed - 0.25 cm/minute; absorption units full scale - 0.1; detection wavelength - 280 nm; run time - 60 minutes. The retention times of both the plasma derived and recombinant 31kD, when run separately, was identical to that obtained for the mixture.

15

Figure 56. Pharmacokinetics of p31 kD in Rats

The figure shows the pharmacokinetics of plasma derived 31 kD in rat serum as a function of time. The rats were injected intravenously with 0.5 mg p31 kD per kg body weight.

20

Figure 57. Binding of ^{125} I-r31 kD to fibrin: effect of thrombin and Ca^{++} ions

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Reaction I was carried out as described in Example 26 using 20 μ l citrated whole blood, 0.15 μ M ^{125} I-r-FBD (r31 kD) (5.6×10^5 cpm/ μ g).

30

Reaction II was initiated with the formation of unlabeled Fibrin clot using 20 μ l citrated whole blood as described in Example 26. After the first incubation, 0.15 μ M ^{125} I-

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r-FBD was added to the existing reaction tube ("Serum") or to the Fibrin pellet following centrifugation and resuspension in PBS ("PBS"). When CaCl_2 and Hirudin were added the concentrations were 5 mM and 3 U/ml, respectively. Reaction II was continued thereafter as described in Example 26.

5 Figure 58. Release of ^{125}I -FBD (p31 kD) from Fibrin clot by plasmin

10 Reaction I was carried out using several tubes containing 100 μl citrated whole blood and 0.3 μM ^{125}I -P-FBD (5.0×10^4 cpm/ μg). At the end of the incubation, the pellet was collected by centrifugation and then resuspended in PBS solution containing 1 $\mu\text{g}/\text{ml}$ plasmin (from porcine blood, Sigma) and was further incubated at 33°C
15 for the indicated time intervals. The reaction was terminated by cooling and immediate centrifugation. The radioactivity in the supernatant and the pellet was measured by a gamma counter.

20 The pellet and the supernatant were resuspended in gel electrophoresis sample buffer (final concentration of 3% glycerol, 2% BME, 1% SDS, 0.2% Bromophenol Blue), boiled for 15 minutes, and electrophoresed in 10% PAGE-SDS. The gel was then autoradiographed on x-ray film. No radioac-
25 tivity was detected from the pellet. Radioactivity from the supernatant was detected in a position corresponding to control ^{125}I -FBD incubated with plasmin (results not shown).

30 Figure 59. The binding of 0.15 μM ^{125}I -r-FBD (^{125}I -r31kD; 5.6×10^5 cpm/ μg) to 20 μl citrated whole blood was performed using reaction I conditions (see Example 26). The reaction was performed either without competitors (C)

or in the presence of various concentrations of unlabeled "folded proteins", "reduced" FBD and related molecules.

rI = "Fully reduced" r31 kD FBD
rII = "Reduced carboxyamidated" r31 kD FBD
p = 31 kD FBD from Trypsin cleavage of plasma
derived Fibronectin.

Figure 60. Binding of ^{125}I -r31 kD to Fibrin (Reaction II). Effect of Transglutaminase inhibitors and related molecules

Unlabeled fibrin clot was formed using 20 μl citrated whole blood and using the conditions described for reaction II in Example 26.

At the end of the first incubation period, and prior to the addition of r- ^{125}I -FBD ($0.15\text{ }\mu\text{M}$; $2.9 \times 10^4\text{ cpm}/\mu\text{g}$), the indicated concentrations of spermidin, putrescine and FBD-"R" ("Reduced-carboxamidated" plasma derived FBD) were added to the specified reactions.

Figure 61. Binding of ^{125}I -r31 kD to preformed fibrin clot (Reaction II): effect of fibrin clot age

The unlabeled fibrin clot was formed using 20 μl citrated whole blood and using the conditions described for reaction II in Example 26.

At the end of the first incubation period, half of the samples were centrifuged and the fibrin pellet was resuspended in PBS solution containing 5 mM CaCl_2 (Reaction "C") or PBS containing 5 mM CaCl_2 and 300 $\mu\text{g}/\text{ml}$ FN (Reaction "D"). No additions or changes were performed to the samples designated "A". 300 $\mu\text{g}/\text{ml}$ FN was added

to the sample designated "B". Subsequently, the reaction mixtures were allowed to incubate at 37°C for 1 hour, 4 hours, or 24 hours (as indicated in the figure). Then ^{125}I -r31 kD (0.15 μM final concentration, 5.4×10^5 cpm/ μg) was added and the incubation was continued for an additional 30 minutes. The reaction was terminated as described in Example 26.

Figure 62. Binding of ^{125}I -r31 kD to "Naive" Thrombi (reactions I and II): effect of thrombi age on the binding

Aliquots of 20 μl non citrated fresh whole human blood were incubated in non siliconized tubes at 37°C with either 0.15 μM ^{125}I -r31 kD or alone (reaction I and II, respectively). At the indicated time intervals, reactions were either terminated (reaction I) or 0.15 μM ^{125}I -r31 kD (2.9×10^4 cpm/ μg) was added, and incubation terminated after an additional 2 hours (reaction II).

Figure 63. Binding of ^{125}I -r31kD to Fibrin (Reaction I): Effect of Exogenous Transglutaminase and "Reduced" FBD

20 μl aliquots of noncitrated whole human blood were incubated with 0.15 μM ^{125}I -r31 kD (2.9×10^4 cpm/ μg) alone ("control") or together with pig liver Transglutaminase ("control + T.G.", 0.2 units/ml; Sigma).

Some of the tubes contained "Reduced" (carboxamidated) p31 kD as indicated in the figure.

The addition of exogenous Transglutaminase to the binding reaction increased the binding values by more than a factor of two. When "reduced-carboxamidated" r31 kD was added to the reaction we observed a similar extent of

inhibitory effect as with the exogenous factor XIIIa (inhibition of 53% and 71% by 0.3 μ M and 3.0 μ M, respectively), indicating an identical inhibitory effect of the reduced FBD on both types of Transglutaminase.

Figure 64. Binding of FBD to ECM

- 5 The biological activity of the FBD was studied in a model of vascular injury as indicated in Example 18, using the Extra Cellular Matrix, "ECM", of cultured endothelial cells.
- 10 33 mm ECM plates following 3x washing in PBS were incubated at 37°C in a CO₂ incubator with 0.5 ml DMEM-10% FCS containing 2.5 mM CaCl₂, 1 μ /ml Thrombin, and the indicated concentrations of ¹²⁵I-P-FBD (about 4.4x10⁵
- 15 cpm/ μ g). Parallel plates were incubated in the absence of thrombin as indicated in the figure. Following 45 minutes of incubation, the plates were washed 3 times with 1 ml PBS, extracted with 0.5 ml of 0.1% SDS-PBS solution, and radioactivity was measured by a gamma
- 20 counter. The values described in the figure represent an average of two plates.

Figure 65. Binding of FN and FBD to S. aureus

- 25 The binding reaction was carried out in a solution using 5x10⁸ PFU/ml of S. aureus SA113 (ATCC Accession No. 35556) and ¹²⁵I-FN (4x10⁴ cpm/ μ g) or ¹²⁵I-FBD (1.3x10⁵ cpm/ μ g; r31 kD FBD, "reoxidized-refolded") at concentrations indicated in the figure and as described in
- 30 methods. The concentration of the labeled molecules described is calculated using molecular weights of 220,000 and 31,000 daltons for FN and r31 kD, respectively.

Figure 66. Binding of FBD to S. aureus: competition
with folded and reduced forms

Binding in solution of 1.25 μg ^{125}I -p31 kD (2.3×10^5 cpm/ μg) to 5×10^8 PFU/ml of S. aureus SA113 was carried out in the presence of the indicated concentrations of the following unlabeled proteins: P-FBD (p31 kD), r-FBD (r31 kD FBD "reoxidized-refolded"), r-FBD-R (r31kD FBD "Reduced Carboxyamidated), and r-CBD (r-33 kD cell binding domain of FN). The binding reaction was carried out as indicated in the methods section.

10

Figure 67. Binding of S. aureus to immobilized FN

Binding of 7.5×10^8 PFU/ml of 3H-leucine-S. aureus (5.8 cpm/ 10^5 PFU) to FN immobilized onto plastic vials was carried out as described in methods and in the presence of human plasma FN, r-FBD (r31 kD FBD "reoxidized-refolded"), P-FBD (p31 kD), or BSA (Bovine Serum Albumin, Sigma). Binding of "control" reaction in the absence of competitors (9.3% of input bacteria) was normalized to 100%.

15
20

Figure 68. Binding of S. aureus to Catheters

Binding of 3.0×10^6 PFU/ml of ^{125}I -S. aureus (1 CPM/3 PFU) to "Uno" bronchial plastic catheters (3 cm for each reaction, in duplicate) coated with FN was carried out as described in methods. When competition reaction was performed, the bacteria and the added protein were pre-incubated at room temperature for 30 minutes and then added to the catheters for further incubation as described in the methods section.

25
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The proteins used in the competition reactions were: P-31 (p31 kD), r-20 (recombinant derived 20 kD FBD) and r-

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31 (reoxidated and refolded r31kD). Some of the reactions (see figure) were measured in the presence of 5 μ m Heparin (from porcine intestinal mucosa, molecular weight of 10,000; Sigma).

5 The binding of "control" reaction in the absence of competitors (8.8% of input bacteria) was normalized to 100%.

Figure 69. Adhesion of p31 kD in Rabbit Aorta Lesion Model

10 The figure shows the distribution of radioactivity in serially sectioned aorta segments from balloon catheterized rabbits. The measurements were taken 72 hours after injection of ^{125} I-labeled Fibronectin (FN) or plasma derived 31kD FBD (31kD).

15 Figure 70. Construction of pTV-170

20 The NdeI-NdeI ApoE fragment was isolated from plasmid pApoE-EX2 (ATCC Accession No. 39787) and inserted into the unique NdeI site of the expression vector p579 (Figure 16) which had been digested with NdeI. The resulting plasmid pTV-170 expresses an analog of natural human ApoE protein having a methionine residue added at the N-terminus.

25 Figure 71. Construction of pTV-194-80

30 The β -lactamase promoter and ribosomal binding site fragment was isolated from plasmid pBLA11 (ATCC Accession No. 39788) after digestion with EcoRI and AluI. This fragment was ligated to the large fragment of pTV-170 (Figure 70) plasmid which had been digested with NdeI, filled in with DNA polymerase I (Klenow) and then digested with EcoRI.

DETAILED DESCRIPTION OF THE INVENTION

5 The plasmids pFN 126-3, pFN 132-5, pFN 975-25, pFN 949-2, and pFN 137-2 were deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession Nos. 67829, 67830, 67832, 67831 and 67910, respectively.

10 The subject invention provides a plasmid for expression of a polypeptide which comprises a substantial portion of the amino acid sequence of one of the domains of naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of
15 naturally-occurring human fibronectin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

20 Also provided is a plasmid for expression of a polypeptide which comprises a substantial portion of the amino acid sequence of the cell binding domain of naturally-occurring human fibronectin and which does not
25 correspond to a product of proteolytic digestion of naturally-occurring human fibronectin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of
30 the polypeptide in a suitable host cell.

Further provided is a plasmid for expression of a polypeptide which comprises a substantial portion of the amino acid sequence of the fibrin binding domain of

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naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

Applicants have provided three examples of fibrin binding domain polypeptide analogs. These include the p31 kD, r31 kD, and r20 kD polypeptides. These polypeptides exhibit the binding and adhesive properties of portions of naturally-occurring human fibronectin. The scope of the claims of the subject application are not intended to be limited to these three FBD analogs, which are examples of preferred embodiments only.

Furthermore, applicants use of the term r31 kD fibrin binding domain polypeptide encompasses the three forms of such protein defined in Example 24 as:

- (a) the protein as it is obtained from a washed pellet, after dissolution in 6 M GuCl, i.e. in "scrambled" form;
- (b) the fully reduced protein, present after treatment with a reducing agent such as GSH in the presence of 6 M GuCl; and
- (c) the reoxidized-refolded protein, obtained by treatment with the GSH/GSSG as described in Example 24.

The "scrambled" r31 kD polypeptide is r31 kD protein which is apparently improperly folded due to the formation of one or more incorrect disulfide bonds.

In preferred embodiments, the polypeptide is about a 75 kD polypeptide fragment of the cell binding domain of human fibronectin, about a 40 kD polypeptide fragment of the cell binding domain of human fibronectin, about a 33 kD polypeptide fragment of the cell binding domain of human fibronectin, about a 31 kD polypeptide fragment of the fibrin binding domain of human fibronectin, or about a 20 kD polypeptide fragment of the fibrin binding domain of human fibronectin.

In more preferred embodiments, the polypeptide is a 75 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1102-1851, but deleted of amino acids 1600-1689; a 40 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1380-1851, but deleted of amino acids 1600-1689; a 33 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1329-1722, but deleted of amino acids 1600-1689; a 31 kD polypeptide fragment of the fibrin binding domain of human fibronectin comprising amino acids 1-262; or a 20 kD polypeptide fragment of the fibrin binding domain of human fibronectin comprising amino acids 1-153 and 13 additional amino acids.

Naturally-occurring human fibronectin is as it occurs in the human body (in plasma).

As used throughout this application, a substantial portion is at least one quarter ($1/4$). A polypeptide which has the biological activity of naturally-occurring human fibronectin exhibits binding or adhesive properties similar to naturally-occurring human fibronectin when the level of such activity is assayed or determined. A polypeptide which has the biological activity of one of

the domains of naturally-occurring human fibronectin, such as the cell binding domain, exhibits binding or adhesive properties similar to the domain of naturally-occurring human fibronectin when the level of such activity is assayed or determined.

5 In this invention, the nucleotide sequences of the various functional domains are determined by cleavage with restriction enzymes, and do not correspond to the nucleotide sequences of the domains as defined by proteolytic digestion of fibronectin.

10 The plasmid of this invention further comprises suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell, such as pro-
15 moters and operators, e.g. λ P_LO_L, ribosomal binding sites, e.g. C_{RII}, and repressors. Other suitable regulatory elements include, for example, the lac, trp, tac, lpp and deo promoters (European Patent Application Publication No. 0303972, published February 22, 1989).

20 The suitable regulatory elements are positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable bacterial host cell. In preferred embodiments of the invention,
25 the regulatory elements are positioned close to and upstream of the DNA encoding the polypeptide.

The invention provides a plasmid designated pFN 126-3 having the restriction map shown in Fig. 10 and deposited
30 in Escherichia coli strain A1645 under ATCC Accession No. 67829. Plasmid pFN 126-3 encodes a 75 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1102-1851, but deleted of amino acids 1600-1689.

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Also provided is a plasmid designated pFN 132-5 having the restriction map shown in Figure 12 and deposited in Escherichia coli strain A4255 (F⁺) under ATCC Accession No. 67830. Plasmid pFN 132-5 encodes a 40 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1380-1851, but deleted of amino acids 1600-1689.

The invention provides a plasmid designated pFN 137-2 having the restriction map shown in Figure 23 and deposited in Escherichia coli strain A4255 under ATCC Accession No. 67910. Plasmid pFN 137-2 encodes a 33 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1329-1722, but deleted of amino acids 1600-1689.

The invention also provides a plasmid designated pFN 975-25 and deposited in Escherichia coli strain A4255 (F⁺) under ATCC Accession No. 67832. Plasmid pFN 975-25 encodes a 31 kD polypeptide fragment of the fibrin binding domain of human fibronectin comprising amino acids 1-262.

Further provided is a plasmid designated pFN 949-2 and deposited in Escherichia coli strain A1645 under ATCC Accession No. 67831. Plasmid pFN 949-2 encodes a 20 kD polypeptide fragment of the fibrin binding domain of human fibronectin comprising amino acids 1-153 and 13 additional amino acids.

The plasmids of this invention may be introduced into suitable bacterial host cells, preferably Escherichia coli. An example of a suitable Escherichia coli cell is strain A4255 (F⁺) [ATCC Accession No. 67830], strain A1645 [ATCC Accession No. 67829], and strain A4255 [ATCC

Accession No. 67910], but other Escherichia coli strains and other bacteria can also be used as host cells for the plasmids. Such bacteria include Pseudomonas aeruginosa and Bacillus subtilis.

5 The bacteria used as hosts may be any strain including auxotrophic (such as A1645), prototrophic (such as A4255), and lytic strains; F^+ and F^- strains; strains harboring the cI^{857} repressor sequence of the prophage (such as A1645 and A4255); and strains deleted for the deo repressors and the deo gene (see European Patent
10 Application Publication No. 0303972, published February 22, 1989). Escherichia coli strain A4255 (F^+) has been deposited harboring plasmids pFN 132-5 and pFN 137-2 under ATCC Accession Nos. 67830 and 67910, respectively. Escherichia coli strain A1645 has been deposited harbor-
15 ing plasmid pFN 126-3 under ATCC Accession No. 67829.

In presently preferred embodiments, the invention provides an Escherichia coli cell containing the plasmid designated pFN 126-3 and wherein the cell and wherein
20 the cell is deposited under ATCC Accession No. 67829.

Also provided is an Escherichia coli cell containing the plasmid designated pFN 132-5 and wherein the cell is deposited under ATCC Accession No. 67830.

25

In a further preferred embodiment, the invention provides a Escherichia coli cell containing the plasmid designated pFN 137-2 and wherein the cell is deposited under ATCC Accession No. 67910.

30

Also provided is a Escherichia coli cell containing the plasmid designated pFN 975-25 and wherein the cell is deposited under ATCC Accession No. 67832.

35

The invention provides a Escherichia coli cell containing the plasmid designated pFN 949-2 and wherein the cell is deposited under ATCC Accession No. 67831.

5 The invention further provides a method of producing a polypeptide which comprises a substantial portion of the amino acid sequence of one of the domains of naturally-occurring human fibronectin which comprises treating an Escherichia coli cell containing a plasmid comprising DNA encoding the polypeptide so that the DNA directs expression of the polypeptide and recovering from the
10 cell the polypeptide so expressed.

Also provided is a method of producing a polypeptide which comprises a substantial portion of the amino acid sequence of the cell binding domain of naturally-occurring human fibronectin which comprises treating an
15 Escherichia coli cell containing a plasmid comprising DNA encoding the polypeptide so that the DNA directs expression of the polypeptide and recovering from the cell the polypeptide so expressed.

20 In addition, the invention provides a method of producing a polypeptide which comprises a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human fibronectin which comprises
25 treating an Escherichia coli cell containing a plasmid comprising DNA encoding the polypeptide so that the DNA directs expression of the polypeptide and recovering from the cell the polypeptide so expressed.

30 Preferably, the polypeptide so produced is a 75 kD, 40 kD, or 33 kD polypeptide of the cell binding domain, or a 31 kD or 20 kD polypeptide of the fibrin binding domain.

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The invention provides a purified polypeptide substantially free of other substances of human origin which comprises a substantial portion of the amino acid sequence of one of the domains of naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin.

Also provided is a purified polypeptide substantially free of other substances of human origin which comprises a substantial portion of the amino acid sequence of the cell binding domain of naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin.

Further provided is a purified polypeptide substantially free of other substances of human origin which comprises a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human fibronectin and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin.

Prefereably, the purified polypeptide is a 75 kD polypeptide substantially free of other substances of human origin which comprises amino acids 1102-1851 of the cell binding domain of naturally-occurring human fibronectin, but deleted of amino acids 1600-1689, which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin.

Another preferred purified polypeptide is a 40 kD polypeptide substantially free of other substances of human origin which comprises amino acids 1380-1851 of the cell binding domain of naturally-occurring human fibro-

nectin, but deleted of amino acids 1600-1689, which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin.

5 The invention also provides a purified polypeptide of 33 kD substantially free of other substances of human origin which comprises amino acids 1329-1722 of the cell binding domain of naturally-occurring human fibronectin, but deleted of amino acids 1600-1689, which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin.

10 Also provided is a purified 31 kD polypeptide of the fibrin binding domain of naturally-occurring human fibronectin substantially free of other substances of human origin which does not correspond to a product of
15 proteolytic digestion of naturally-occurring human fibronectin, and a purified 20 kD polypeptide of the fibrin binding domain of naturally-occurring human fibronectin substantially free of other substances of human origin which does not correspond to a product of
20 proteolytic digestion of naturally-occurring human fibronectin.

The invention also provides a bacterially-produced polypeptide which comprises a substantial portion of the
25 amino acid sequence of one of the domains of naturally-occurring human fibronectin which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin. Preferably, the domain is the cell binding domain or the fibrin binding domain of naturally-occurring human fibronectin. Preferred
30 polypeptides include: a bacterially-produced 75 kD polypeptide which comprises amino acids 1102-1851 of the cell binding domain of naturally-occurring human fibronectin, but deleted of amino acids 1600-1689 which does

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not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin; a bacterially-produced 40 kD polypeptide which comprises amino acids 1380-1851 of the cell binding domain of naturally-occurring human fibronectin, but deleted of amino acids 1600-1689 which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin; a bacterially-produced 33 kD polypeptide which comprises amino acids 1329-1722 of the cell binding domain of naturally-occurring human fibronectin, but deleted of amino acids 1600-1689 which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin; a bacterially-produced 31 kD polypeptide of the fibrin binding domain of naturally-occurring human fibronectin which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin; and a bacterially-produced 20 kD polypeptide of the fibrin binding domain of naturally-occurring human fibronectin which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin.

The invention provides a polypeptide which comprises a substantial portion of the amino acid sequence of one of the domains of naturally-occurring human fibronectin which does not correspond to a product of proteolytic digestion of human fibronectin. The domain preferably is the cell binding domain or the fibrin binding domain. Preferably, the polypeptide is a 75 kD polypeptide comprising amino acids 1102-1851, but deleted of amino acids 1600-1689, a 40 kD polypeptide comprising amino acids 1380-1851, but deleted of amino acids 1600-1689, a 33 kD polypeptide comprising amino acids 1329-1722, but deleted of amino acids 1600-1689, a 31 kD polypeptide comprising amino acids 1-262, or a 20 kD polypeptide

comprising amino acids 1-153 and 13 additional amino acids.

5 The invention provides a composition comprising at least two of the polypeptides disclosed above and a suitable carrier. The polypeptides in such a composition may be bound to one another.

10 As used throughout the subject application, "bound" encompasses polypeptides bound covalently, non-covalently, or conjugated. The polypeptides may be conjugated through other chemical moities including amino acid or polypeptide linkers, which are standardly used in the art and are well-known to those skilled in the art to which the subject invention pertains.

15 Also provided is a hybrid polypeptide consisting essentially of at least two of the polypeptides disclosed above.

20 The invention also provides a pharmaceutical composition comprising an amount of the composition comprising at least two of the polypeptides effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier and a pharmaceutical composition comprising an amount of the hybrid polypeptide effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier.

30 Further provided is a pharmaceutical composition comprising an amount of any one of the disclosed polypeptides effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier, and a pharmaceutical composition comprising an amount of any of the disclosed polypeptides effective to inhibit thromboxane release from platelets and a pharmaceutically acceptable carrier.

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The invention also provides a method of inhibiting platelet aggregation which comprises contacting platelets under suitable conditions with an amount of any of the disclosed polypeptides effective to inhibit platelet aggregation, and a method of inhibiting thromboxane release from platelets which comprises contacting
5 platelets under suitable conditions with an amount of any of the disclosed polypeptides effective to inhibit thromboxane release from the platelets.

10 The invention further provides a method of treating a subject with a cerebrovascular or cardiovascular disorder which comprises administering to the subject an amount of any of the disclosed polypeptides effective to inhibit platelet aggregation.

15 Examples of cardiovascular disorders susceptible to treating include acute myocardial infarction, or angina. Subjects who have undergone angioplasty or coronary bypass surgery or who have received thrombolytic treatment may also be treated with the polypeptides of the
20 subject invention so as to inhibit platelet aggregation.

The invention provides a method of treating a subject with a wound which comprises administering to the subject
25 an amount of any of the disclosed polypeptides effective to promote healing of the wound.

The wound may be a cutaneous wound, such as an incisional, a skin deficit, a skin graft or a burn wound.
30 The wound may also be an eye wound, such as a corneal epithelial wound or a corneal stromal wound, or a tendon injury.

This invention further provides a method of treating a subject susceptible to, or afflicted with, a bacterial infection which comprises administering to the subject an amount of any of the disclosed polypeptides effective to prevent or treat the bacterial infection. Such a bacterial infection may be due to the presence of a catheter or an implant in the subject.

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The invention also provides a method of treating a subject with cancer which comprises administering to the subject an amount of any of the disclosed polypeptides effective to retard tumor metastasis, and a method of
10 detecting a tumor in a subject which comprises administering to the subject an amount of any of the disclosed polypeptides effective to detect the tumor.

The polypeptides of the subject invention can also be
15 used in a method of detecting a thrombus in a subject which comprises administering to the subject an amount of the polypeptide effective to detect the thrombus.

In each of these detection methods, for tumor and
20 thrombus, the polypeptide is used as a diagnostic agent for detecting the tumor or thrombus. Numerous methods are known in the art for such detection, such as radioactive labeling (nuclear medicine use of isotopes), radio-opaque labeling (such as CAT scan), and Magnetic Reso-
25 nance Imaging (MRI). Any of these labeling methods can be used in the method of the subject invention for detecting the tumor or thrombus.

The invention also provides any of the disclosed polypep-
30 tides bound to a thrombolytic agent. The thrombolytic agent is selected from the group consisting of: tissue plasminogen activator (TPA), urokinase, streptokinase,

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prourokinase, Anisoylated Plasminogen-Streptokinase Activator Complex (EminaseTM), or TPA analogs.

5 The invention provides any of the disclosed polypeptides bound to a growth factor, such as: EGF, PDGF; α -TGF, β -TGF, FDGF, TNF, interleukins, interferons, erythropoietin, colony-stimulating factor (CSF), GM-CSF, G-CSF, or CSF-I. The polypeptides may also be bound to serum albumin, a blood factor such as Factor VIII or Factor XIII, polyethyleneglycol, or to superoxide dismutase.

10 The invention provides a coated medical device comprising a medical device and the polypeptides of the fibrin binding domain of naturally-occurring human fibronectin applied as a coating to the surface of the medical device. Examples of medical devices which may be coated include catheters, medical implants (such a hip replace-
15 ment and prostheses), tubings and syringes.

20 The invention provides a method of minimizing risk of bacterial infection associated with use of medical devices which comprises:

- (a) applying the polypeptide of the fibrin binding domain of fibronectin as a coating to a surface of the device; and
- 25 (b) employing the resulting coated device rather than an uncoated device.

30 Applicants' invention also provides uses of tryptic fragments of the fibrin binding domain. Such tryptic fragments are obtained by proteolytic digestion of plasma derived fibronectin. These tryptic fragments may be used in a method of inhibiting platelet aggregation which comprises contacting platelets under suitable conditions

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with an amount of the polypeptide effective to inhibit platelet aggregation, such polypeptide comprising a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human fibronectin.

5 Further provided is a method of inhibiting thromboxane release from platelets which comprises contacting platelets under suitable conditions with an amount of a polypeptide effective to inhibit thromboxane release from the platelets, such polypeptide comprising a substantial
10 portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human fibronectin.

Also provided is a method of treating a subject with a cerebrovascular disorder which comprises administering
15 to the subject an amount of a polypeptide effective to inhibit platelet aggregation, such polypeptide comprising a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human fibronectin.

20 The polypeptide of the fibrin binding domain may be used to treat a subject with a cardiovascular disorder which comprises administering to the subject an amount of a polypeptide effective to inhibit platelet aggregation,
25 such polypeptide comprising a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human fibronectin. The cardiovascular disorder may include acute myocardial infarction or angina. Subjects who have undergone angioplasty or coronary bypass surgery or who have received thrombolytic
30 treatment may also be treated with the plasma-derived fibrin binding domain polypeptides.

The invention also provides a method of treating a subject with a wound which comprises administering to the subject an amount of a fibrin binding domain polypeptide effective to promote healing of the wound, such polypeptide comprising a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-
5 occurring human fibronectin.

The wound may be a cutaneous wound, such as an incisional wound, a skin deficit wound, a skin graft or a burn wound. The wound may also be an eye wound, such as a
10 corneal epithelial wound or a corneal stromal wound, or a tendon injury.

The invention also provides a method of treating a subject susceptible to, or afflicted with, a bacterial
15 infection which comprises administering to the subject an amount of a polypeptide effective to prevent or treat the bacterial infection, such polypeptide comprising a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human
20 fibronectin. The bacterial infection may be due to the presence of a catheter or an implant in the subject.

Further provided is a method of treating a subject with cancer which comprises administering to the subject an
25 amount of the fibrin binding domain polypeptide effective to retard tumor metastasis, such polypeptide comprising a substantial portion of the amino acid sequence of the fibrin binding domain of naturally-occurring human fibronectin.

30 The invention provides a method of detecting a tumor in a subject which comprises administering to the subject an amount of a FBD polypeptide effective to detect the tumor, such polypeptide comprising a substantial portion
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of the amino acid sequence of the fibrin binding domain
of naturally-occurring human fibronectin, and a method
of detecting a thrombus in a subject which comprises
administering to the subject an amount of such a polypep-
tide effective to detect the thrombus, such polypeptide
comprising a substantial portion of the amino acid
5 sequence of the fibrin binding domain of naturally-
occurring human fibronectin.

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EXAMPLES

All the references to map positions correspond to the identically numbered positions along the nucleotide sequence of human fibronectin cDNA shown in Figure 41
5 (see also Figure 3 of Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1987).

The cDNA sequence applicants have cloned and expressed is missing the 270 bp extra domain (ED) segment which
10 extends from nucleotides 4811 to 5080, inclusive, on the Baralle map (see Figure 41). Thus, the cDNA sequence which is said to extend from nucleotide 3317 to 5566 on the Baralle map, contains only 1980 nucleotides, because it is missing the 270 nucleotides of the ED segment,
15 namely from nucleotides 4811 to 5080 inclusive; this region is also known in the art as the ED-A region. Similarly, the protein expressed by that DNA fragment would encode from amino acid 1102 to amino acid 1851 on the Baralle map but would be missing the 90 amino acids
20 encoded by the ED region, namely amino acids 1600-1689 inclusive, and thus it would contain only 660 amino acids. This is true for all fragments described in this application which span the ED region. (The region known in the art as the ED-B region is missing both in
25 Baralle's sequence and in applicants' cDNA.)

The EcoRI cleavage site shown at position 3317 was constructed by applicants during the cloning procedure by use of EcoRI linkers. This GAATTC sequence at
30 positions 3313 to 3318 differs in 1 nucleotide from the corresponding Baralle sequence GATTC. This introduces a single nucleotide change C to A at nucleotide 3315. This changes the corresponding amino acid from Thr to Asn.

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EXAMPLE 15 Preparation of a Fibronectin cDNA Library

A cDNA library was prepared in gt11 from poly A+ mRNA isolated from human liver according to the published procedures (13,14). The cDNA fragments were cloned using
 10 EcoRI linkers and the cDNA library was screened for fibronectin (FN) positive plasmids using the following synthetic DNA probes.

Probes for cell binding domain (CBD):

15	<u>Probe</u>	<u>Nucleotides</u>
	(3') CACTCTATAATGTCCTAGTGAATGCCTCTTTGTCCTCC	(4355- 4392)
20	(3') AGAATCTCCTTCTGTCTTTTGTCCAGAACTAAG	(3967- 3999)
	(3') CCGGTTGTTAGTTGTCAAAGACTACAAGGCTCCCTGGACC	(4200- 4239)

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Probes for N-terminal domain:

	(3') GGGGGTCGGAGGGATACCGGTGACACAGTGTCTTAA	(817-850)
30	(3') CGACGGGTGCTCCTTTAGACGTGTTGGTTACTTCCCCAGTAC	(1310- 1340)

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A series of FN cDNA clones covering the entire region of fibrin, collagen, heparin and cell binding domains was identified and isolated (Figure 1). The cDNA fragments were subcloned into the EcoRI site of pBR322.

The mRNA of FN is alternatively spliced and therefore different length cDNA's have been reported in the literature. Applicants' cDNA has a 270 base pair deletion from base 4811 to base 5080 on the FN physical map (the complete non spliced cDNA).

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EXAMPLE 2Expression of the CBD in Escherichia coli

5 In order to obtain expression of the 75kD and 40kD Cell Binding domain (CBD) proteins of FN, various partial cDNA clones were constructed from DNA fragments derived from different FN cDNA clones, described in Example 1.

10 A. Subcloning of the 5' end of CBD

A synthetic oligomer with the sequence

15 5' TATGGATCAATTC 3'
ACCTAGTTAAGTTAA

was ligated to an EcoRI-BamHI fragment derived from cDNA clone pFN919 (bases 3317 to 4151) to create an NdeI site.
20 The ligated fragment was inserted into NdeI-BamHI digested pBR322 (Figure 2).

The plasmid obtained was designated pFN100. The plasmid cloning was carried out in Escherichia coli strain MC
25 1061.

A synthetic oligomer with the sequence

30 TATGGATCAGAGCT
ACCTAGTC

was ligated to a SstI-BamHI fragment derived from cDNA clone pFN919 (bases 3611 to 4090) to create an NdeI site.

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The ligated fragment was inserted into NdeI-BamHI digested pBR322 (Figure 3).

5 The plasmid obtained was designated pFN101 and was maintained in Escherichia coli strain MC 1061.

B. Subcloning of the 3' end of CBD

10 A synthetic oligomer containing a termination codon and HindIII and SalI restriction sites was ligated to the 3' end of an EcoRI-BglII fragment derived from cDNA clone pFN916 (bases 4151 to 5566). The ligated fragment was inserted into an EcoRI-SalI digested pBR322 plasmid
15 (Figure 4). The plasmid obtained was designated pFN102 and was maintained in Escherichia coli strain MC 1061.

The synthetic oligomer had the following sequence:

20 5' GATCTTAAGCTTG 3'
AATTCGAACAGCT

C. Subcloning of the entire CBD

25 In order to obtain a full size CBD subclone having an NdeI site (including an initiation codon ATG) at the 5' end and a HindIII (including a termination codon) at the 3' end (Figure 3), plasmid pFN102 was digested with EcoRI and HindIII enzymes, and the 3' end EcoRI-HindIII of the
30 CBD was isolated and ligated to plasmid pFN100 which had been digested with BamHI and HindIII (Figure 5). The ligation was performed in the presence of a BamHI-EcoRI synthetic fragment with the sequence shown in Figure 15.

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The plasmid obtained was designated pFN103 and was maintained in Escherichia coli strain MC 1061.

5 D. Expression of the entire CBD

In order to obtain expression of the entire CBD in Escherichia coli an NdeI-HindIII fragment was isolated from plasmid pFN103 and ligated into plasmid pTV301
10 digested with NdeI and HindIII (Figure 6).

The plasmid obtained was designated pFN105 and was maintained in Escherichia coli strain A 1645.

15 Plasmid pTV301 was constructed as shown in Figure 7 by NdeI cleavage of human growth hormone (hGH) expressor plasmid pTV104(2). Plasmid pTV104(2) has been deposited with the ATCC under Accession No. 39384. The NdeI-NdeI hGH fragment was inserted into the NdeI site of expression vector plasmid p579. Plasmid p579 was constructed
20 from plasmids pR0211 and pPS1 (Figure 16). Plasmid pPS1 has been deposited with the ATCC under Accession No. 39807. Plasmid pR0211 was constructed from plasmid pJH200 (Figure 17). Plasmid pJH200 has been deposited
25 with the ATCC under Accession No. 39783.

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EXAMPLE 3Subcloning and Expression of Part of the Cell Binding
Domain (CBD)

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In order to obtain expression of the CBD protein of approximately 65 kD containing the RGD sequence, plasmid pFN102 was digested with EcoRI-HindIII, and the fragment containing the CBD was isolated and ligated to plasmid pFN101 in the presence of a BamHI-EcoRI synthetic fragment with the sequence shown in Figure 15 (See Example 2) (Figure 8). The plasmid obtained was designated pFN104 and was maintained in Escherichia coli strain MC 1061.

15

To obtain expression of the CBD in Escherichia coli an NdeI-HindIII fragment of FN was isolated from plasmid pFN104 and ligated into plasmid pTV301 digested with NdeI and HindIII (Figure 9). The plasmid obtained was designated pFN106 and was maintained in Escherichia coli strain A 1645.

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EXAMPLE 45 Protein Analysis of Expressed Proteins

Plasmids pFN105 and pFN106 were used to transform
Escherichia coli strain A1645. The clones obtained were
grown in LB medium containing Ampicillin (100 µg/ml) at
10 30°C to O.D. of 0.7. Expression was obtained upon
induction at 42°C for 1-3 hours.

Bacterial cells after induction were harvested and
centrifuged for 5 minutes at 10,000 RPM. Pellets were
15 suspended in ten volumes of 50 mM Tris-HCl pH=8.0/0.5 mM
EDTA, 1mM PMSF (i.e. if the pellet weighs one gram, then
10 volumes of solution are added). Sample buffer
(containing SDS and β-mercaptoethanol) was added.
Samples were boiled for 10 minutes and then spun in
20 microfuge at top speed for 2 minutes; 15 µl of the
resulting supernatant were loaded onto a 10% SDS poly-
acrylamide gel. The expected sizes of the entire and
partial CBD clones were 75 kD and 65 kD respectively.
However, the proteins obtained upon induction were
25 truncated and had the size of 45 kD and 35 kD respective-
ly.

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EXAMPLE 55 Construction of Plasmids Expressing Non-Truncated Proteins

In order to clarify why truncated proteins were obtained, the DNA of clones pFN105 and pFN106 was resequenced. A
10 deletion of one nucleotide, a T at position 4030, was observed. In order to overcome this obstacle the original cDNA library was rescreened for additional cDNA clones which hybridize to the FN cell binding domain probes. Positive cDNA clones were isolated and the DNA
15 sequenced. A cDNA clone designated pFN114-4 (bp 3573-6720) was found to have the correct DNA sequence.

Expression of a Non-Truncated 75 kD Protein

20 In order to obtain a non-truncated CBD protein, the SstI-BglII fragment of clone pFN105 was replaced by an SstI-BglII fragment of cDNA clone pFN114-4 (Figure 10). Plasmid pFN114-4 is an additional plasmid harboring FN cDNA isolated independently from the cDNA library;
25 pFN114-4 has no deletion of the thymidine in position 4030. An SstI-BglII fragment from plasmid pFN114-4 was used to replace the SstI-BglII fragment from plasmid pFN105. The resulting plasmid p117-4 directed the expression of the full length 75kD cell binding domain
30 protein but the plasmid does not contain the translation termination codon and terminates within the pBR322 sequence. To correct this fault, the following linker was constructed:

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GATCTACTAAGGCCTA

ATGATTCCGGATTCTGA

5 This linker contains BglII and HindIII sites at the 5' and 3' terminals respectively, and codes for the 3' end of the 75 kD CBD including the translation termination codon. It was ligated to plasmid pFN117-4 which had been cleaved with BglII and HindIII (Figure 10). The resulting plasmid pFN 126-3 directed the expression of the full
10 length 75 kD cell binding domain protein, with the authentic C-terminus. Plasmid pFN 126-3 has been deposited in Escherichia coli A1645 with the American Type Culture Collection under Accession No. 67829. The
15 protein expressed has the amino acids met-asp-gln-phe-asn-ser (MDQFNS) added to the amino terminus of a protein which begins at amino acid 1102 and extends to amino acid 1851 of the fibronectin molecule (minus amino acids 1600-1689). This molecule differs from the 75 kD protein
20 obtained by partial tryptic digests of fibronectin. The tryptic polypeptide fragment extends from amino acid no. 873 to amino acid no. 1555.

Expression of a non-truncated partial 40kD CBD protein

25 Plasmid pFN102 was digested by EcoRI and HindIII. The FN EcoRI-HindIII fragment was isolated and ligated to plasmid pTV301 which had been digested with NdeI and HindIII in the presence of a synthetic DNA fragment
30 having the sequence:

TATGGATCAATTC

ACCTAGTTAAGTTAA

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The resulting plasmid designated pFN118, and maintained in Escherichia coli strain A1645, expressed a 40 kD CBD protein. Due to a mistake in the synthesis of the BglIII-HindIII termination linker this protein was not terminated at the BglIII site but in the DNA sequence originating from pBR322 (Figure 11).

In order to correctly terminate the translation, plasmid pFN118 was digested by BglII and NdeI, and the BglII-NdeI FN fragment was isolated and ligated to both the NdeI-HindIII large fragment isolated from plasmid pTV301 and also to a synthetic linker (Figure 12). The synthetic linker had the following sequence:

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GATCTACTAAGGCCTA

ATGATTCCGGATTCTGA

The plasmid obtained, pFN132-5, was found to express the 40kD CBD protein which terminated at the correct site. The original transformation of plasmid pFN132-5 was carried out in Escherichia coli strain A 1645. After plasmid analysis (restriction enzymes, DNA sequencing and determination of the 40 kD protein expression) the plasmid was then used to transform Escherichia coli strain 4255 (F⁺). Plasmid pFN 132-5 has been deposited in Escherichia coli with the American Type Culture Collection under Accession No. 67830. The protein expressed has the amino acids met-asp-gln-phe (MDQF) added to the amino terminus of a polypeptide which begins at fibronectin amino acid 1380 and extends to amino acid 1851 of the fibronectin molecule (minus amino acids 1600-1689). This molecule differs from the 75 kD protein obtained by partial tryptic digests of fibronectin. The

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tryptic polypeptide fragment extends from amino acid no. 873 to amino acid no. 1555.

5 Growth conditions

The clone pFN132-5 in Escherichia coli strain 4255 (F⁺), expressing the 40 kD FN CBD, was grown in LB medium containing ampicillin to an O.D. of 0.7 at 30°C. Expression was obtained upon induction at 42°C for 1-3 hours.

Purification of the 40kD CBD protein

15 Cells were harvested after induction at 42°C for 2 hours, centrifuged and five grams of pelleted material were suspended in 50ml buffer (50mM Tris-HCl pH=8.0, 5mM EDTA, 1mM PMSF, 25mM NaCl) and sonicated 5 times (1 minute each) at 4°C. After centrifugation the 40kD CBD protein was found to be in the pellet fraction.

The 40 kD CBD protein was further purified by solubilization of the pellet in 6M Urea (in the above buffer) and fractionation by DEAE cellulose ion exchange column chromatography. The 40kD protein was pooled and dialyzed against PBS. An improved purification method for the 40kD protein is disclosed in Example 15.

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EXAMPLE 6Cell Binding Activity of the 40 kD CBD Polypeptide

5 The purified 40kD CBD was assayed for biological activity. Authentic plasma FN was used as a positive control. Plasma FN and recombinant 40 kD CBD were immobilized on a plastic surface. The binding of
10 Tritium-labeled mouse 3T3 cells to the layer was determined as a function of the immobilized proteins concentration, as described by Pierschbacher, M., et al. (15, 18). Results indicate that the extent of cell binding by the 40 kD protein is comparable to that of the
15 authentic plasma FN. Additional results are disclosed in Example 16.

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EXAMPLE 7Platelet Aggregation Assay

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To evaluate the ability of different fibronectin recombinant fragments to inhibit platelet aggregation, experiments were performed in an in vitro assay in the presence of plasma proteins.

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Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP) (16, 17) was as follows:

1. To 20 mls of human blood at room temperature, 2mls of 4.8% sodium citrate were added.

15

2. After centrifugation for 10 minutes at 1,200 RPM in a Beckman centrifuge at 20°C the supernatant (PRP) was collected.

20

3. 1 ml of the above supernatant was further centrifuged at 10,000 RPM for 2 minutes at room temperature, and the supernatant (PPP) was separated.

25 Assay

The aggregometer was calibrated on 0.1 and 0.9 transmittance units using the PRP and PPP, respectively.

30 Reaction tube mixture

- a. 125 μ l of PRP.
- b. 0 μ l-125 μ l protein sample in PBS solution.
- c. equilibrate solution to 37°C for 2-3 minutes.

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d. final reaction volume 250 μ l, adding PBS to final volume.

5 Platelet aggregation was induced by adding an ADP solution to a final concentration of 10 μ M (5 μ l of 0.5mM ADP solution). The results are summarized in Table I and demonstrate that both the 40 kD CBD and the 31 kD FBD proteins partially inhibit the ADP induced platelet
10 aggregation, while undigested, whole plasmatc fibronectin had no effect.

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TABLE I

		<u>Activity</u>	<u>Inhibition</u>
5	complete system	%	%
	(5 μ M ADP) 125 μ l PRP	100	0
10	+ <u>GRGDS</u> (synthetic pentameric peptide)		
	500 μ M		100
	250 μ M		100
	100 μ M		100
	50 μ M	20	80
	25 μ M	40	60
15	12.5 μ M	51	49
	+ <u>r-40 kD CBD</u>		
	2.5 μ M	37	63
	2.5 μ M	28	72
	1.25 μ M	42	58
20	0.625 μ M	82	18
	+ <u>31 kD FBD</u> (purified from fibronectin)		
	4 μ M	0	100
	2 μ M	9	91
25	0.5 μ M	62	38
	+ <u>plasma purified fibronectin</u>		
	0.5 μ M	110	0
30	+ <u>recombinant produced met APO-E</u> (negative control)		
	4 μ M	100	0

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TABLE II

**Effect of Applicant's recombinant proteins
on platelet aggregation**

		Protein Concentration (μ M)	Inhibition of aggregation %
5			
10			
	plasma purified fibronectin	0.5	0
15	degraded plasma fibronectin	0.5	50
	purified plasma 31 kD FBD	0.8	40
20	GRGDS (pentapeptide)	50	60
	40kD rCBD	0.5	40
25	recombinant Apolipoprotein E	4	0
	recombinant bovine growth hormone (bGH)	22	0

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The activity of the rCBD domain in preventing ADP-induced aggregation of platelets clearly demonstrates that this polypeptide is an efficacious inhibitor : 40% inhibition was obtained with 0.5 μ M of the purified polypeptide (Table II). Plasma-purified, intact fibronectin did not have any effect under the assay conditions. In contrast, the same preparation which underwent limited proteolysis, to yield distinct fibronectin domains, gained a strong inhibitory activity.

The RGD-containing synthetic pentapeptide, at relatively high molar concentrations, exhibited an inhibitory effect, as reported in the literature. Two proteins, recombinant bGH and recombinant Apolipoprotein E, were tested as negative controls, and as expected did not prevent platelet aggregation. The purified plasma FBD inhibits platelet aggregation in vitro. This finding will be further corroborated using recombinant FBD. More results showing the effect of the 40 kD protein on platelet aggregation are disclosed in Example 17.

EXAMPLE 8Isolation of 31 kD Fibrin Binding Domain and Demonstration of Its Anti-Aggregating Effect on PlateletsSummary

A 31 kD tryptic fragment from plasmatric FN was isolated and purified by chromatography on DEAE-cellulose, CM-Sepharose* and Heparin-Sepharose*. The purified fragment was found to be effective in preventing platelet aggregation (40% inhibition at concentrations between 0.5 and 1.1 μ M).

Preparation and purification of the fragment

The 31 kD fragment was obtained by cleavage of plasmatric FN (purified on a Gelatin-Sepharose* column from which it was eluted and stored in 1M guanidinium hydrochloride). Thus, 206 mgs of FN - after dialysis against 10 mM of Tris-HCl - were digested with 0.01% of TPCK-trypsin at 37°C for 5 min. The tryptic digest was loaded on a DE52 column (6ml) and 1/5 of the flow-through fraction (50 ml) was applied on a CM-Sepharose column (3 ml) and eluted with a NaCl gradient (0-0.5 M). About 80% of the protein was recovered in the salt gradient (peak at about 220mM) and after dialysis to remove the salt about 1/2 of the protein was loaded on a Heparin-Sepharose* column (1.5 ml) and eluted with 0.5 M NaCl. Approximately 75% of the protein was recovered in this fraction, i.e., about 1 mg (about 40% of the theoretical yield). This fraction was >90% pure 31 kD fragment, and was found to prevent platelet aggregation in vitro.

* Trademark

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Prevention of Platelet Aggregation In-Vitro

5 Using human PRP and ADP (10 μ M)-induced aggregation, two separate experiments were performed in which the results were as follows:

	<u>Conc. of p31 kD (μM)</u>	<u>% Inhibition</u>	<u>Expt. #</u>
10	4.0	100	1
	2.0	91	
	0.5	38	
	1.1	39	2
15	0.3	12	

Characterization

20 The purified fragment was characterized by SDS-PAGE under reducing and non-reducing conditions, by gel filtration chromatography on Superose 12 (molecular weight 26 kD) and by N-terminal sequencing (the N-terminus was found to be blocked, as expected for a pyroglutamate residue).
25 Furthermore, the material did react in immunoblots with anti-FN and anti-rec20 kD antibodies. (Rec 20 kD represents a recombinant FN molecule which contains amino acids 1-190.) Its binding characteristics to Heparin-Sepharose also constitute evidence for its nature.
30 This 31 kD fragment also reacted with Staphylococcus aureus in a bacteria binding assay.

EXAMPLE 9Expression and Purification of Fibrin Binding Domain
5 (FBD) AnalogsA. Expression of a partial FBD 20 kD protein

10 The FN cDNA clones obtained as described in Example 1 and depicted in Figure 1, did not include DNA encoding amino acids 1-190 of the FN molecule. These amino acids are part of the FBD. The DNA corresponding to nucleotides 14 to 472 and coding for amino acids 1 - 153 (Figure 41A) was constructed by ligation of 7 pairs of chemically synthesized nucleotides (Figures 42 and 43). The
15 synthetic DNA fragment was designed to contain an ATG initiation codon at the 5' end as well as convenient restriction sites for introduction into various expression vectors. To enable further manipulation of the DNA sequence coding for the FBD, nucleotide number 19, thymidine(T) was changed to adenine (A), thereby eliminating a DdeI restriction site without altering the amino acid sequence. (The site of the nucleotide change is denoted by an asterisk in linker #1 shown in Figure 42A.) The
20 various steps for the cloning of the above synthetic DNA fragment into pBR322 plasmid vector digested with EcoRI and BamHI are described in Figure 43. The plasmid obtained was designated pFN 932-18. The DNA fragment coding for the first 153 N-terminal amino acids of fibronectin from plasmid pFN 932-18, was inserted into
30 pTV 301, a λ P_L expression vector, between the NdeI and BglIII sites replacing the DNA sequence coding for human growth hormone (hGH) in plasmid pTV 301 (Figure 44).

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The resulting plasmid, pFN 949-2, was deposited with the American Type Culture Collection under Accession No. 67831. Plasmid pFN 949-2 was used to transform Es-
5 cherichia coli prototroph A4255. These transformed Es-
cherichia coli cells were found to express the partial FBD protein in amounts comprising about 5% of the total cellular proteins. The protein has a mobility of about 20 kD on reduced SDS polyacrylamide gels as determined from the mobility of the size markers. The protein
10 comprises methionine followed by the first 153 amino acids of fibronectin followed by 4 amino acids coded for by a synthetic linker and then 9 amino acids resulting from readthrough into the pBR322 vector, i.e. a total of 167 amino acids. Throughout this specification the
15 protein is referred to as the r20 kD protein or the r20 kD FBD.

B. Expression of a "complete" FBD protein analog

20 In order to obtain expression of the entire FBD protein containing amino acids 1 to 262 the following plasmids were constructed:

25 1. Insertion of termination codon TAA at the 3' end

A synthetic oligonucleotide containing a TAA termination codon and a BglIII site having the following sequence:

30 5' CTGTTTAAGCA
3' GACAAATTCGTCTAG

was ligated to the 3' end of an EcoRI-PvuII fragment isolated from FN cDNA clone p931-5 and to a pBR322 vector

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digested with EcoRI and BamHI as described in Figure 45. The plasmid obtained was designated pFN935-12.

5 2. Subcloning of carboxy terminal region of FBD in a λ P_L expression vector

10 An EcoRI-HincII DNA fragment coding for the carboxy terminal region of the FBD was isolated from plasmid pFN935-12 and ligated to plasmid pTV 194-80 digested with EcoRI and SmaI as described in Figure 46. The plasmid obtained was designated pFN 946-12.

15 3. Syntheses and cloning of DNA corresponding to nucleotides 468-599 of FN

20 Three pairs of chemically synthesized nucleotides were ligated to an EcoRI-DdeI FN fragment isolated from plasmid pFN932-18 (Figure 43) in the presence of pUC19 vector DNA (purchased from GIBCO BRL Co.) digested with EcoRI and XbaI as described in detail in Figure 47. The plasmid obtained was designated pFN 948-4.

25 4. Construction of a plasmid encoding the complete FBD region

30 In order to construct a plasmid which codes for the entire FBD, amino acid 1 to amino acid 262, an EcoRI-XbaI DNA fragment coding for FN was isolated from plasmid pFN948-4 and inserted into plasmid pFN 946-12 digested with EcoRI and XbaI as described in Figure 48. The plasmid obtained was designated pFN-957. This plasmid contains the complete coding sequence for FBD but does

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not express the FBD protein as it lacks a ribosomal binding site (RBS).

5
5. Expression of the FBD under λP_L promoter and cII RBS

10 An NdeI-HindIII fragment containing the FBD coding region and the T_1T_2 transcription terminators was isolated from plasmid pFN-957 and inserted into plasmid pTV 301 (Figure 7) digested with NdeI and HindIII as described in Figure 49. The resulting plasmid, designated as pFN 962-3, directs the expression of a FBD analog protein under the control of λP_L promoter and cII ribosomal binding site.
15 Escherichia coli strains A1645 and A4255 transformed with this plasmid expressed only small amounts of the FBD protein. The expression of the FBD protein was detectable only by Western blot analysis using polyclonal
20 antibodies directed against human plasma derived FN.

6. Expression of an FBD analog protein under the λP_L promoter and the β -lactamase promoter and ribosomal binding site

25 As the level of expression of the FBD analog protein obtained with plasmid pFN 962-3 was low, we added a DNA fragment coding for the β -lactamase promoter and β -lactamase RBS (PBLA). The DNA fragment coding for PBLA
30 was isolated from plasmid pBLA11 (ATCC No. 39788) and inserted into plasmid pFN 962-3 digested with NdeI, filled in with Klenow enzyme and digested with EcoRI as described in Figure 50. The plasmid obtained, designated pFN 975-25, was deposited with the American Type Culture

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Collection under Accession No. 67832. This plasmid was used to transform Escherichia coli prototroph A4255 (F+).

5 These Escherichia coli cells were found to express the "complete" FBD analog protein at levels comprising about 5-8% of the total cellular proteins. The protein migrated on SDS-PAGE gels with an apparent molecular weight of 31 kD, hence it is referred to as the 31 kD
10 protein or the r31 kD FBD.

C. Fermentation and growth conditions

15 The clone expressing the r31 kD FBD protein was fermented in rich medium (yeast extract and casein hydrolysate) containing ampicillin. Growth was carried out at 30°C. Expression was obtained upon induction at 42°C for 2 hours.

20 D. Refolding and purification of recombinant Fibrin Binding Domain (r31kD) protein

The process is made up of three stages:

- 25
1. Crude processing of the bacterial cake
 2. Refolding/reoxidation
 3. Purification

1. Crude processing

30 The cake is disrupted first in 5 volumes of 50mM Tris-HCl/50 mM Na-EDTA, pH 8 (Buffer 1); the pellet is then treated with 1.2 volumes of Buffer 1 containing 100 mg/liter lysozyme (2 hours agitation at 37°C). Triton

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X 100^{*} is added to the resulting suspension (to 1%), and after 30 min. at room temperature the suspension is centrifuged and the pellet is resuspended and washed twice with water. All these steps are performed by
5 disruption of the pellet and centrifugation and the 31 kD stays in the pellet, as evidenced from SDS-PAGE gels.

The washed pellet is suspended in 14 volumes of 10 mM Tris-HCl/5 mM EDTA/2mM PMSF/2mM 6-aminocaproate, pH 7.5
10 (Buffer A) and then treated successively with Buffer A containing: 1% decyl sulfate, 1% decyl sulfate/5% glycerol and 5% glycerol. The final treatment is with Buffer A without additives.

15 2. Refolding/reoxidation

Principle: To dissolve the pellet in 6M guanidine-HCl - GuCl - in the presence of a thiol reducing agent, such as glutathione - GSH - and to refold/reoxidize at a lower
20 GuCl concentration by the addition of oxidized glutathione-GSSG.

The washed pellet from step 1 above is dissolved in 150-700 volumes of 6M GuCl/3mM GSH in Buffer A. The
25 concentration of GuCl is lowered gradually, i.e., first 2 M, then 1 M and 0.5 M, while keeping the concentration of all other components constant, except for the volume, which at this stage is brought to 500-1000 fold higher than that of the pellet. At one of the intermediate
30 concentrations of GuCl, i.e. between 0.5 and 2 M, refolding is initiated by the addition of 0.3 mM of GSSG and incubation at room temperature for 24-48 hours. The

* Trademark

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refolded 31 kD is then dialyzed against Buffer A without additives.

5 3. Purification

Concentration: The large volume of refolded 31 kD is first centrifuged to remove the insoluble pellet that contains no 31 kD and is then dialyzed against Tris-HCl, pH 7.8, before being concentrated and initially purified
10 on a Heparin-Sepharose column.

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EXAMPLE 10Wound Healing

5 The r40 kD CBD fragment domain was tested in a rat wound
healing model. In this model, a polyvinyl sponge is
implanted subcutaneously, and test substances are
injected in situ. The effect of the test materials, in
10 terms of chemotaxis and extracellular matrix formation,
is reflected by increased DNA and collagen accumulation
in the sponge (see Figure 13).

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EXAMPLE 11Bacterial Binding Activity

5 Experiments have been performed on the binding of r31 kD
FBD to bacterial suspensions of Staphylococcus aureus.
Identical binding curves were obtained for radio-iodin-
ated intact plasma fibronectin, the proteolytic 31 kD
10 amino terminal fragment (p31 kD) derived from human
plasma fibronectin, and r31 kD FBD.

The inhibition of bacterial binding by [¹²⁵I]p31 kD
indicated that the recombinant 31 kD FBD competes with
15 the authentic proteolytic fragment.

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EXAMPLE 12Inhibition of Bacteria Adhesion

5 To estimate the capacity of r31 kD FBD to interfere with
the adherence of bacteria to the extracellular matrix in
wounds, a competition assay was developed. In this
assay, adherence of Staphylococcus aureus to a plastic
10 surface coated with fibronectin and the interference of
FBD with adherence were measured. Both authentic FBD and
r31 kD FBD were active in inhibiting bacterial adhesion
to the fibronectin coated surface.

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EXAMPLE 13Pharmacokinetics of the Recombinant 40 kD Cell Binding
5 Domain of Fibronectin

The pharmacokinetic behavior of the 40 kD recombinant protein was studied in rats. Purified 40 kD CBD was labeled with radioactive iodine and injected intravenously at a dose of 1 mg/kg body weight. Blood levels of radioactivity followed an initial decline during the first hour with a half-life of about 1h, followed by a slower phase of clearance during 1-8 h with a longer half-life ($t_{1/2} = 3.2h$). Hence, the duration of the recombinant protein in the body is relatively long (see Figure 14).

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In comparison, the half-life of the RGD peptide is 8 minutes.

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EXAMPLE 14Construction of Expression Vectors for Recombinant
Proteins Corresponding to Various Regions of the Cell
Binding Domain, and Expression of These Recombinant
Proteins

The vectors described below for expression of various regions of the CBD were all constructed from the plasmids encoding 75 kD and 40 kD proteins described in Examples 2-5. Figure 18 shows diagrammatically the length and location of the cDNA in each plasmid and indicates the presence or absence of cDNA encoding Cell Binding Domains I and II (CBD I and CBD II). Figure 19 summarizes information about the proteins expressed by the resulting plasmids.

Expression of the proteins described below was achieved in all cases (unless otherwise indicated) as follows. The plasmids were originally used to transform Escherichia coli strain A1645 and, after plasmid analysis (restriction enzyme data and determination of expression), the plasmids were then used to transform Escherichia coli strain A4255 (F^+).

In order to construct a plasmid expressing a protein similar to the 75 kD protein but with a truncated C-terminus, expression plasmid pFN 128-4 was constructed as shown in Figure 20. This plasmid expresses a protein with a molecular weight as measured by polyacrylamide gel electrophoresis of 65 kD; this protein was therefore designated "the 65 kD protein". The 65 kD protein is

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expected to be 531 amino acids long as defined in Figure 19.

5 A plasmid expressing a protein with deletion of even more of the C-terminal section (including deletion of the CBD I region) than plasmid pFN 128-4 was constructed as shown in Figure 21. The resulting plasmid, pFN 130-11, expresses a protein with a molecular weight as measured
10 on SDS-polyacrylamide gel electrophoresis of 28 kD. This recombinant protein, designated "the 28 kD protein", is expected to contain 258 amino acids as defined in Figure 19.

15 To produce a recombinant protein similar to the 65 kD protein but truncated at the N-terminus, expression plasmid pFN 143-1 was constructed as shown in Figures 22-24. This plasmid expresses a protein with a molecular weight of 55 kD as measured by SDS-polyacrylamide gel
20 electrophoresis. The protein, designated "the 55 kD protein", is expected to contain 436 amino acids as defined in Figure 19.

In order to produce a recombinant protein similar to the
25 75 kD protein but with deletion of the N-terminal region containing the CBD II region, expression plasmid pFN 135-12 was constructed as shown in Figure 22. Both the parent plasmid (pFN 117-4) and this daughter plasmid (pFN 135-12) contain the same 3' terminus, which lacks a
30 translation termination codon. Thus, translation terminates within the pBR322 mRNA, and the resulting protein contains at its C-terminus additional non-fibronectin derived amino acids.

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The molecular weight of the protein expressed, as measured on SDS polyacrylamide gels, was 45 kD, and the protein was therefore designated "the 45 kD protein".
5 Including the non-fibronectin derived C-terminus it is expected to contain approximately 433 amino acids as defined in Figure 19.

To produce a protein similar to the 45 kD protein but
10 with a truncated C-terminus, plasmid pFN 137-2 was constructed as shown in Figure 23. Plasmid pFN 137-2, in Escherichia coli host A4255, was deposited with the ATCC under Accession No. 67910. This plasmid expresses a protein with a molecular weight of 33 kD as measured
15 by SDS-polyacrylamide gel electrophoresis, and this protein was therefore designated "the 33 kD protein". It is expected to contain 304 amino acids as defined in Figure 19.

20 In order to produce a recombinant protein similar to the 33 kD protein but truncated at the N-terminus, expression plasmid pFN 134-9 was constructed as shown in Figure 25. This plasmid expresses a protein with a molecular weight of 28 kD as measured by SDS-polyacrylamide gel electro-
25 phoresis. This protein was designated "the 28(*) kD protein". The 28(*) kD protein is expected to contain 253 amino acids as defined in Figure 19.

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EXAMPLE 15Purification of the 40 kD and 33 kD Proteins

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(a) Purification of the 40 kD protein

The 40 kD protein, produced as described in Example 5, can be purified as described for the 40 kD protein on the last page of Example 5. Alternatively, it was purified
10 by the modified method described below.

10g of wet bacterial cake was resuspended in 100 ml of Buffer A solution:

15

50 mM Tris - HCl pH = 8.0

5 mM EDTA

25 mM NaCl

1 mM PMSF

20

10 mM ethylenediamine

25

The resulting suspension was sonicated for 8 minutes in ice, and this sonication was repeated twice. The resulting sonicate was centrifuged for 30 minutes at 15,000 rpm at 4°C.

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The resulting bacterial pellet was resuspended in 100 ml Buffer A containing 6M urea and sonicated for 4 minutes. The sonicate was centrifuged for 30 minutes at 15,000 rpm and 500 OD units of the resulting supernatant solution was loaded on a DE52 cellulose column (250 ml) equilibrated with Buffer A. The column was washed with Buffer A and fractions were collected. The purified 40 kD protein (Pool I+II) eluted within 1/6 of the flow

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through volume. This preparation of recombinant protein was then dialyzed against Buffer A and purified 40 kD protein fractions were pooled and dialyzed against PBS.

5 Subsequently, large scale preparations of the 40 kD protein were purified, essentially as described above, except that a DEAE-Sepharose Fast Flow column (Pharmacia) replaced the DE52 cellulose column. The partially
10 purified 40 kD protein was then further purified from high-molecular weight aggregates and from lower molecular weight contaminants by size exclusion chromatography on a Fractogel® TSK HW-55 (F) column (E. Merck), which had been treated for pyrogen removal according to specifica-
15 tions. Typically, 100 ml of the partially purified 40 kD protein, which had been concentrated to about 4 mg/ml by ammonium sulfate precipitation and redissolved in 6 M urea in Buffer A, was loaded on the Fractogel® TSK 55 column and eluted in Buffer A in the absence of urea.

20 The pool of purified 40 kD protein, which was stored frozen, had a concentration of 1.6 mg/ml and was greater than 92% pure.

25 (b) Purification of the 33 kD protein

Escherichia coli strain A4255 (F⁺) harboring plasmid pFN 137-2 which expresses the 33 kD protein was grown in LB medium containing ampicillin at 30°C. Expression was
30 obtained upon induction at 42°C for 1-3 hours. The wet bacterial cake was sonicated as described in (a) above. A washed bacterial pellet (70 g) was dissolved in 1600 ml of freshly deionized 6M urea in Buffer A (as described above).

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Following centrifugation to eliminate insoluble material, which contained negligible amounts of the 33 kD protein, two chromatographic runs were performed on a 10 x 63 cm DEAE-Sepharose Fast Flow (Pharmacia) column, which had been previously equilibrated with the same buffer. Elution was performed with Buffer A in the absence of urea. Under these conditions the 33 kD fragment is retarded on the column, whereas most of the contaminant bacterial proteins bind and are eluted from the column on addition of 0.5 M NaCl to the buffer. Fractions were pooled according to SDS-PAGE profiles of the column and resulting pools of the purified fractions of the two runs were combined (DEAE-S 3, 4). The protein concentrations of these pools were determined by Bradford's modified method (24), and the purity was evaluated from Coomassie Brilliant Blue staining of SDS-PAGE profiles. The results are summarized in Table III.

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TABLE III

5	<u>Step</u>	<u>Protein</u> <u>conc. (mg/ml)</u>	<u>Total</u> <u>protein (mg)</u>	<u>Purity</u> <u>(SDS-PAGE)</u>
	6M urea solubilization	5.5	8740	(35%)
10	DEAE-S 3, 4			
	pool 1	0.93	1674	(92%)
	pool 2	0.55	990	(85%)

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EXAMPLE 16Effect of the 33 kD and 40 kD Proteins in a Cell Attachment System

The effect of the 33 kD and 40 kD proteins on cell attachment was studied using a slightly modified cell attachment assay (23) (see also 15, 18). Preliminary results are described in Example 6. Briefly, immobilization of the test protein ligand in PBS solution to a plastic surface takes place at 4°C for 16 hours. After washing out the unbound protein with PBS and blocking non-specific sites with BSA in PBS, ³H-thymidine labeled trypsinized growing eucaryotic cells are laid on the surface and incubated at 37°C for 45 min.

After washing, the radioactive label remaining on the dishes is measured. This is performed at various concentrations of test protein ligand to produce a dose-response curve, as shown in Figures 38 and 39.

Surprisingly, the 40 kD protein binds to 3T3 fibroblasts and BHK cells in a similar manner to plasmatc fibronectin (although it does not bind to NRK cells). However, the 33 kD protein binds to BHK cells very poorly. The 40 kD protein appears to bind to the integrin receptors which are present on the cell surface of the eucaryotic cells analyzed, since additional experiments show that its binding was completely inhibited by the pentapeptide GRGDS, similarly to that of the plasmatc fibronectin.

It is unexpected that the 40 kD protein is involved in cell attachment in a similar degree to intact fibronectin.

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tin, since it does not contain the CBD II site believed to work in a synergistic manner with the CBD I (RGD) site (see 25). The two-thirds of the heparin binding site present in the 40 kD protein (see Figure 1) may be serving, unexpectedly, a cell-attachment function, and may compensate for the missing CBD II site. This hypothesis is substantiated by additional cell-attachment experiments which demonstrate that heparin competes with the binding of the 40 kD protein, but not with the binding of fibronectin.

Note that the 33 kD and the 40 kD proteins both contain the CBD I region but not the CBD II region (see Figure 18). The 40 kD protein is extended at the C-terminus (by 387 base pairs = 129 amino acids) and this region contains two-thirds of the heparin binding domain, which is not present in the 33 kD protein.

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EXAMPLE 17Effect of the 40 kD and 33 kD proteins in a platelet
aggregation assay

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The in vitro assay described in Example 7 was used to evaluate the ability of the 40 kD and 33 kD proteins to inhibit platelet aggregation. The ability of the 40 kD protein to partially inhibit ADP-induced platelet aggregation was shown in Example 7 (Tables I and II).

10

The dose-response effect of the 33 kD protein, the 40 kD protein, plasmatic 75 kD and the pentapeptide GRGDS in the in vitro platelet aggregation assay system is shown in Figure 28. These results, using PRP, show that all the proteins tested inhibit platelet aggregation but that the 33 kD protein has the strongest effect.

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A summary of similar experiments is shown in Table IV; plasmatic 75 kD is prepared as described in the Description to Figure 28.

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TABLE IV

Effect of Applicants' Recombinant Proteins in an
in vitro Platelet Aggregation Assay Using PRP

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<u>Test protein/peptide</u>	<u>Concentration to</u> <u>achieve 50%</u> <u>inhibition</u>	
	<u>μM</u>	<u>mg/l</u>
33 kD protein	1-3	33-100
40 kD protein	4-6	168-248
Plasmatic 75 kD	5-8	375-600
Synthetic Pentapeptide		
15 GRGDS (Sigma)	50-100	33-66

Note that only 33-100 mg per liter of the 33 kD protein
is needed to achieve 50% inhibition of aggregation.

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Similar in vitro aggregation studies using PRP were performed to examine the effect of the 33 kD protein compared to that of the 40 kD protein (before and after urea treatment, as described in Figure 20). The results
5 show that urea treatment of the 40 kD protein increases its inhibitory effect on platelet aggregation, and that the 33 kD protein has even greater inhibitory effect.

10 The inhibition of platelet aggregation by the 40 kD and 33 kD proteins in whole blood was examined in the same in vitro assay system as described above and modified as described in the Description to Figure 29. The graphs of percent inhibition as a function of protein concentra-
15 tion are shown in Figure 29.

These results show that the 33 kD and 40 kD proteins inhibit platelet aggregation in whole human blood, and that the inhibitory effect of the 33 kD protein is
20 surprisingly strong. 80% inhibition is achieved using 1.5 μ M of the 33 kD protein or 2.5 μ M of the 40 kD protein; this is equivalent to 50 mg/liter of the 33 kD protein and 100 mg/l of the 40 kD protein.

25 The 33 kD protein has also been tested to determine whether it can prevent aggregation of collagen-induced platelets. Experiments were performed, using the same platelet aggregation assay system as described above, but inducing with 8 μ g/ml collagen (Type I, Sigma) instead
30 of ADP. The 33 kD protein inhibited the aggregation of platelets by collagen. The effective dose of the 33 kD protein needed to prevent aggregation is about 5-10 fold higher than for ADP-stimulated cells. Similar results were obtained using PRP from rats.

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The effect of the 40 kD and 33 kD polypeptides and the GRGDS polypeptide on the aggregation of platelets in different species was studied. PRP was prepared from blood from rats, rabbits, guinea pigs, dogs and humans and the inhibition of aggregation was measured using the aggregation assay described above. The results are shown in Table V.

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TABLE V

Effects of the 40 kD and 33 kD proteins and the GRGDS polypeptide on aggregation of platelets in different species

	Compound Tested (μ M)	Inhibition of Aggregation (%)
10		
	Rat	
	GRGDS (200)	10
	33 kD (6)	100
15	40 kD (12)	100
	Rabbit	
	GRGDS (200)	50
	33 kD (2)	75
	40 kD (6)	60
20	Guinea pig	
	GRGDS (400)	75
	33 kD (7)	70
	40 kD (12)	70
25	Dog	
	GRGDS (50)	70
	33 kD (3)	90
	40 kD (6)	80
30	Human	
	GRGDS (75)	50
	33 kD (1.5)	50
	40 kD (5)	50

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Table V shows that the 33 kD and 40 kD proteins, which are homologous to human fibronectin, inhibit aggregation of platelets from different mammalian species. The average concentrations needed for 50-100% inhibition of platelet aggregation is comparatively much lower than that of GRGDS, demonstrating the efficacy of these recombinant fibronectin fragments in blocking platelet function.

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EXAMPLE 18Effect of the 40 kD and 33 kD proteins in a model of
5 vascular injury

The biological activity of the 40 kD and 33 kD proteins was studied in a model of vascular injury (26). Briefly, cultured endothelial cells produce an extracellular
10 matrix (ECM) which activates platelets, similarly to de-endothelialized vascular segments. The incubation of human platelets on these ECMs induces platelet adhesion, aggregation, thromboxane A₂ (TXA₂) formation and a release reaction.

15 When platelet-rich plasma (PRP) is incubated on ECM-coated plates, large platelet aggregates are formed. The interaction of the platelets in this model system is monitored by phase contrast microscopy and by measurement
20 of thromboxane B₂ (TXB₂) by radioimmunoassay, as described by Eldor et al. (26). Note that unstable thromboxane A₂ (TXA₂) is released first and is rapidly converted to thromboxane B₂ (TXB₂). This model system can be used to test various agents which interfere with
25 platelet adhesion, aggregation and release.

The effect of the GRGDS pentapeptide and the 40 kD and 33 kD proteins was studied in this model system. 1 ml of PRP mixture, containing 300 μl PRP and 700 μl PBS, was
30 applied in the presence or absence of the test compounds, to culture dishes coated with ECM. After incubation for 45 minutes, the size of platelet aggregates and TXB₂ released was determined. Small aggregates (+) covered an area of $1-2 \times 10^3 \mu^2$ while large aggregates (++++)

covered an area of $1-2.5 \times 10^5 \mu^2$. Other details of the assay system are described by Eldor et al. (26).

The results are shown in Table VI.

TABLE VI

Effect of Applicants' Recombinant Proteins
on Platelet Activation and Aggregation
in a Model of Vascular Injury

	Platelet Aggregation (microscopic)	Thromboxane B2 (ng/ml)	Inhibition of Thromboxane release (%)
Control	++++	49.6	0
75 μ M			
GRGDS peptide	+	37.1	25
14 μ M			
40 kD protein	+	4.0	92
14 μ M			
33 kD protein	+	2.7	96

These results demonstrate that the 40 kD and 33 kD proteins inhibit platelet aggregation strongly and nearly completely. Additionally, they almost completely inhibit thromboxane release (92% and 96% respectively); in striking contrast, the GRGDS pentapeptide inhibits TXB₂

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release only marginally (25%). Moreover, microscopic analysis of the ECM plates in the presence of the 33 kD protein revealed that most of the platelets were in suspension and not attached to the ECM, indicating that
5 the 33 kD protein specifically affects platelet adhesion to the ECM; this effect was less pronounced with the 40 kD protein.

10 In summary, the 33 kD protein binds specifically in a time- and dose-dependent manner to resting and activated platelets; it also inhibits platelet aggregation in solution as well as on ECM surfaces and inhibits platelet adhesion to the ECM as well as thromboxane B₂ release.
15 This surprising combination of properties demonstrates the uniqueness of the 33 kD protein and its utility as an anti-thrombotic drug.

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EXAMPLE 19Binding of the 40 kD and 33 kD recombinant proteins to
5 platelets

The in vitro binding of the 40 kD and 33 kD recombinant proteins to platelets was studied.

10 METHOD:

The binding of the recombinant proteins to platelets was assayed as described by Ginsberg et al. (27) with the following modifications. The Sepharose 2B column was not
15 used; instead, 5 ml of 24 hour concentrated platelets from the Israel Blood Bank (approximately 2×10^9 platelets/ml) was centrifuged for 10 min at 1,500 rpm in a Sorvall centrifuge. The resulting pellet, resuspended in a 1:7 solution of ACD:Saline Buffer (see below), was
20 centrifuged again for 10 min. at 1,500 rpm in a Sorvall centrifuge. The resulting pellet was resuspended in 2.5 ml Tyrode's buffer (see below) and the number of platelets was counted (approximately 4×10^9 platelets/ml); this platelet preparation was designated "washed plate-
25 lets" and was used in all of the platelet binding experiments described.

The reaction mixture contained:

- 30 a) 133.3 μ l washed platelets (5×10^8 platelets).
b) 10-600 nM ^{125} I-labeled protein.
c) unlabeled protein as indicated.
d) 1 unit thrombin (as stimulus, unless otherwise indicated).

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e) PBS to final reaction volume (200 μ l)

10 μ l of the above reaction mixture was removed for
measurement of total radioactivity. The remainder was
5 incubated at 37°C for 30 minutes (unless otherwise
indicated). In triplicate, 50 μ l of the reaction mixture
was placed in a siliconized microfuge tube containing 1
ml of 20% sucrose 0.65% BSA/Tyrode's buffer.

10 After centrifugation for 3 minutes at top speed in the
microfuge tube the solution was aspirated and the
radioactivity of the pellet containing the platelets was
measured in a gamma counter. This indicates the amount
of radioactive protein bound to the platelets.
15

ACD Buffer: 8 g/L citric acid
22 g/L sodium citrate
2 g/L glucose

20 Tyrode's Buffer: 5 mM Hepes pH = 7.5
0.137M NaCl
2.7 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
2 mM CaCl_2
3.8 mM NaH_2PO_4
25 Glucose 1 g/L
BSA 3.5 g/L

RESULTS:

30 The results of binding experiments using the above assay
are shown in Figures 31-38, and described in the Descrip-
tion of the Figures. Figures 31 and 35 show that

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40,000-125,000 molecules of the 33 kD or the 40 kD proteins bind per platelet.

5 The binding of the recombinant 40 kD and 33 kD proteins to platelets is abolished by the addition of either cold homologous protein or the synthetic pentapeptide GRGDS (Sigma) (Figure 32) and these results are similar to those obtained using plasma FN. These results indicate
10 that the 40 kD and 33 kD recombinant proteins bind specifically to the platelet receptor sites, which GRGDS recognizes; these receptors are known as integrins.

The binding of the recombinant 40 kD and 33 kD proteins to thrombin-stimulated and unstimulated platelets was
15 studied (Figure 33). These results showed that the 33 kD protein was able to bind to unstimulated platelets whereas the 40 kD protein binds to unstimulated platelets at a much lower level. Additionally, these results
20 showed again that unlabeled homologous proteins compete with the labeled 40 kD and 33 kD proteins; this specific competition also occurred when the 33 kD protein was bound to unstimulated platelets.

25 The effect of the recombinant 40 kD and 33 kD proteins on the binding of fibrinogen to thrombin-stimulated and unstimulated platelets was examined (Figure 34). These experiments were performed at 25°C in the presence of hirudin to prevent the conversion of fibrinogen to fibrin
30 by thrombin. The results demonstrated that the 40 kD and 33 kD proteins compete with fibrinogen in platelet binding. The GRGDS pentapeptide similarly competed with fibrinogen in platelet binding, indicating once again that the 40 kD and 33 kD proteins bind to the platelet,

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and compete with the major platelet ligand (fibrinogen), probably at the GRGDS recognition site.

5 The dose-response effect of the binding of the 40 kD and 33 kD recombinant proteins to platelets was studied (Figure 35). The 40 kD protein binds well only when the platelets are thrombin-stimulated (Figure 35A), whereas the 33 kD protein will bind to unstimulated platelets to a level of up to 50% of the maximal binding achieved with
10 unstimulated platelets.

Time courses of the binding of the 33 kD protein to stimulated and unstimulated platelets gave similar results (Figures 36 and 37). The 33 kD protein binding
15 to unstimulated platelets is about 80% of its level of binding to stimulated platelets; i.e., the 33 kD protein binds to platelets almost independent of their activation status. These experiments also demonstrated that most of this binding takes place within 10 minutes of mixing
20 of the reactants.

Further studies in the binding of the 40 kD and 33 kD proteins to platelets were performed in the presence of murine monoclonal antibody to the GPIIb/IIIa receptor
25 (Dakopatt a/s, Denmark). Binding of the proteins to platelets was measured as described above. Results indicate that the 40 kD and 33 kD proteins may bind to stimulated platelets at the GPIIb/IIIa site, whereas the 33 kD protein may bind to unstimulated platelets at some
30 other RGD-dependent site of the integrin superfamily.

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EXAMPLE 20Reactivation of the 40 kD recombinant protein by urea

5 The 40 kD protein becomes inactive in the antiplatelet aggregation assay on storage due to aggregation. Reactivation can be achieved by the following procedure.

- 10 i) Concentrate the solution containing the 40 kD protein using 50% ammonium sulfate to an OD₂₈₀ of 2.0-3.0 at 4°C while stirring.
- 15 ii) Centrifuge the resulting solution at 4°C for 30 minutes at 10,000 rpm.
- iii) Resuspend the resulting pellet in buffer A containing 6M urea, freshly treated with AG-501-x8, an analytical grade mixed bed resin (Biorad).

20

Buffer A

- 50 mM Tris - HCl, pH = 8.0
5 mM EDTA
25 25 mM NaCl
1 mM PMSF
10 mM Ethylenediamine

This can be left at 4°C for 3-16 hours.

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- iv) The resulting solution is dialyzed gradually against 100 x volume of the following solutions:

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- a) 3 changes of buffer A containing 3 M urea. (Total of 2-3 hours.)
- 5 b) 3 changes of buffer A containing 1.5 M urea. (Total of 2-3 hours.)
- c) 3 changes of buffer A containing 0.5 M urea. (Total of 2-3 hours).
- 10 d) 3 changes of buffer A. (Total of 3-16 hours.)
- e) 2 changes of PBS.
- 15 f) 2 changes of PBS in "reverse osmosis" (RO) water.

The total dialysis time is about 48 hours.

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EXAMPLE 21Characterization of the 33 kD and the 40 kD proteins5 a) Preliminary characterization of the 33 kD protein

10 The 33 kD protein was purified as described in Example 15, and characterized as follows. The 33 kD protein has a coefficient of absorption, based on a protein concentration determined by the modified Bradford method (see Example 15), of $E_{1\%}^{1\text{cm}}=16.2$ at 280 nm and a ratio of absorbancies (R) between the spectrum's maximum (at 278 nm) and the minimum (at 252 nm) of $R=2.3$.

15 The major fraction (about 80%, according to the 280 nm absorption) of the size exclusion profile was run on a FPLC equipped with a Superose 12 column (Pharmacia) and displayed an apparent molecular weight of 47 kD. The rest of the absorption is distributed as follows: up to 20 10% consists of a high molecular weight (approximately 10^3 kD) aggregate and about 10% consists of a low molecular weight (less than 10 kD) component. The 33 kD fragment displays a U-shaped solubility curve with a minimum solubility between pH values 3.5 and 6.5.

25

Amino acid sequence

30 The N-terminal sequence of the 33 kD protein was found to be Met-Ser-Pro-Thr-Gly; the Escherichia coli methionine aminopeptidase does not remove the additional methionine residue derived from the ATG coding sequence.

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b) Preliminary characterization of the 40 kD protein

5 The 40 kD protein was purified as described in Example 15 to greater than 92% purity and it was then characterized as follows.

10 The absorption spectrum of the 40 kD protein was determined; it has a coefficient of absorption, based on a protein concentration determined as in (a) above, of $E_{1\%}^{1\text{cm}}=15.9$ at 280 nm and a ratio of absorbancies (R) between the spectrum's maximum (at 278 nm) and the minimum (at 250 nm) of $R=2.3$.

15 About 60% of the 40 kD protein preparation consists of an oligomeric form of molecular weight smaller than 300 kD, and greater than 40% of the preparation consists of a soluble-aggregate form of molecular weight approximately 1000 kD, as determined from FPLC runs on a Superose
20 12 column (Pharmacia). However, this aggregation was found to be reversible in 4M urea, since under these conditions the 40 kD protein elutes from the Superose 12 column (in 4M urea) as a monomer.

25 Amino acid sequence

30 The N-terminal sequence of the 40 kD protein was found to be Met-Asp-Gln-Phe-Asn-Ser-Ile-Thr-Leu-Thr. The Met-Asp-Gln-Phe sequence is added to the N-terminus of the fibronectin domain, which begins at the subsequent Asn (see Figure 19).

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EXAMPLE 22In vivo sponge implant wound healing system

5 The effect of the 40 kD protein on wound healing in vivo was studied using sponge implants. Preliminary results are described in Example 10.

10 METHOD

Purified 40 kD protein, prepared as described in Examples 5 and 15, was combined with isotonic (285 m osmoles), sterile pyrogen-free saline at a final concentration of 0.2-0.5 mg/ml. Sterilized polyvinylalcohol sponge disks, IvalonTM (Unipoint Industries, N. Carolina), were surgically implanted subcutaneously, under the dorsal skin of (6-8 week old) female Sprague-Dawley rats (weighing 150-200 g). A single longitudinal incision 2-3 cm long was made along the midline of the anesthetized animals, penetrating the full thickness of the shaved, disinfected skin. Through this incision four sponges (10 mm) were inserted and positioned beneath the panniculus carnosus at a distance 1-2 cm from the incision and 2-3 cm from each other.

Each animal was implanted with four sterile sponges and analysis of each experimental condition was performed on identically treated sponges from individual rats. Five days after implantation the animals were lightly anesthetized and the sponges were injected with 0.1 ml of one of the following test materials: 0.9% saline alone; the 40 kD protein in saline (0.5 mg/ml); native fibronectin purified from human plasma, stored in guanidine hydro-

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chloride and dialyzed against three changes of PBS before use (1 mg/ml in saline); and bovine serum albumin (1 mg/ml in saline). Animals were sacrificed 48 hours after injection. Implanted sponges were dissected free of loosely adherent fat and muscle, and weighed (wet weight). Sponges were assayed for DNA, protein and collagen content. One sponge from each animal was fixed in buffered formalin and histological sections were prepared and stained with Masson's trichrome to reveal cellular and matrix elements.

To determine DNA and protein content, the sponges were homogenized in 1N NH_4OH and extracted overnight at 4°C. DNA content was determined according to the method of Burton (29). Protein content was measured according to the method of Lowry. Hydroxyproline content as a measure of collagen content was determined by the method of Woessner (30), after acid hydrolysis of the sponge in vacuo (6N HCl at 110°C for 6 hours).

RESULTS

The results shown in Figure 13 demonstrate the increase in the DNA and collagen content of sponges treated with the 40 kD protein compared to saline-treated or BSA-treated sponges. The DNA and collagen levels were significantly elevated in 40 kD protein-treated sponges ($p < 0.01$) but not in BSA-treated sponges.

The results shown in Figure 40 indicate that the sponges treated with the 40 kD protein had elevated DNA and collagen levels, in a similar manner to sponges treated with plasmatc fibronectin (FN), a positive control.

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Epidermal growth factor, another positive control, displayed a similar effect.

5 Pharmacokinetics

10 The pharmacokinetics of the radioactively labeled 40 kD protein were studied in the rat, and the results of intravenous injection are shown in Figure 14. The half life of the 40 kD protein after intravenous injection is 3.2 hours and after intraperitoneal injection is 4.2 hours.

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EXAMPLE 23

The effect of the 33 kD protein on ex-vivo platelet aggregation

5 Preliminary experiments using the 33 kD protein in an
ex-vivo system were performed. The material tested is
injected intravenously into rodents, and PRP prepared
from blood samples (taken at various times
post-injection) are tested for platelet aggregation in
10 vitro. The method used is as described in Example 7.
In rats, each animal can supply only a single sample, and
control animals are used as a basis for comparison. In
control animals an equal volume of phosphate buffered
saline is injected instead of the protein. In rabbits
15 each animal can be used for multiple samples and a time
course of the action of the test material can be es-
tablished.

In the rat experiments 3-15 mg/kg of the 33 kD protein
was injected, and in some experiments the 33 kD protein
20 inhibited platelet aggregation completely in response to
an ADP challenge, at times ranging from 10-15 minutes
post injection. However, highly varying degrees of
inhibition were found necessitating further study.

25 In a limited number of trials using rabbits, inhibitory
effects of the 33 kD protein were observed up to 1/2 hour
post-injection.

30 These results give further support to the potential of
the 33 kD protein as an anti-thrombotic drug.

Pharmacokinetics

The pharmacokinetics of radioactively-labeled 33 kD protein after intravenous injection in rats was studied. The results are shown in Figure 27. The half-life of the⁵ 33 kD protein in blood after intravenous injection was found to be 40 minutes.

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EXAMPLE 24

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Refolding and purification of recombinant 31 kD fibrin-binding fragment of fibronectin

10 The following is an improved procedure for the purification of the recombinant 31 kD fibrin-binding domain (r31 kD) produced as described in Example 9.

The process is made up of three stages:

- 15 1. Crude processing of the bacterial cake.
2. Refolding/reoxidation.
3. Purification.

1. Crude processing

20

1.1 Washing of the pellet: The bacterial cell cake is disrupted first in 5 volumes of 50 mM Tris-HCl/50 mM Na-EDTA, pH 8 (Buffer 1). The pellet is then successively treated with Buffer 1 containing 100 mg/liter lysozyme (2 hours at 37°C), Buffer 1 containing 1% Triton X-100 (30 minutes at room temperature) and twice with water. All these steps are performed by disruption of the pellet and centrifugation; the r31 kD stays in the pellet, as evidenced from SDS-PAGE gels.

30

1.2 Extraction of the pellet: The washed pellet is suspended in 14 volumes of 10 mM Tris-HCl/5 mM EDTA/2 mM PMSF/2 mM

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5 epsilon-aminocaproate (Buffer A) pH 7.5, and then treated successively with Buffer A containing: (a) 1% decyl sulfate; (b) 1% decyl sulfate/5% glycerol; and (c) 5% glycerol. The final treatment is with Buffer A without additives.

10 2. Refolding/reoxidation

A refolding/reoxidation procedure for the recombinant 31 kD fragment (r31 kD) has been developed and refined.

15 2.1 Principle: To dissolve the pellet in 6 M guanidine-HCl (GuCl) in the presence of a thiol reducing agent, such as glutathione (GSH) and to refold/reoxidize at a lower GuCl concentration by the addition of oxidized glutathione (GSSG).

20 2.2 Procedure: The extracted pellet is dissolved in 100-700 volumes of 6 M GuCl/3 mM GSH in Buffer A, pH 8.0. The concentration of GuCl in the dialysis buffer is lowered gradually, e.g., first 3 M, then 1.5 M and
25 finally 0.5 M, while keeping the concentration of all other components constant. At one of the intermediate concentrations of GuCl, i.e., between 2 M and 1 M, refolding is initiated by the addition of 0.3 mM of GSSG and incubation at pH 8 at room temperature for 48-72
30 hours. The refolded r31 kD is then dialyzed against Buffer A at pH 8.5, without additives.

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5 Example: Approximately 10 grams of extracted pellet (see
1.2) were homogenized and dissolved in 1 liter of Buffer
A/6 M GuCl/3 mM GSH/pH 8 and the suspension was stirred
for 14 hours until it was a clear solution. This
10 solution was dialyzed for 24 hours against four liters
of Buffer A which additionally contained 3 mM GSH and 3
M GuCl, pH 8. Subsequently the resulting solution was
dialyzed for 24 hours against 8 liters of Buffer A
containing 3 mM GSH, pH 8. The resulting solution was
dialyzed twice against 10 liters of Buffer A containing
15 0.3 mM GSH and 0.3 mM GSSG, pH 8. The process of
dialysis, during which reoxidation also occurred, lasted
approximately 80 hours. Finally, the glutathione was
removed from the refolded protein by dialysis against 10
liters of Buffer A, pH 8.3 - 8.5. This step was per-
formed twice. Subsequently the solution was loaded on
20 a phenyl-Sepharose column.

2.3 Alternative procedure: Similar results have been
obtained when cysteine (3 mM) was used instead of
glutathione and cystine (0.3 mM) instead of oxidized
25 glutathione.

2.4 Use of thioredoxin: Attempts were also made to increase
the rate of reoxidation of the r31 kD, by using thiore-
doxin. Based on SDS-PAGE profiles run in the absence of
mercaptoethanol (ME), thioredoxin reduction and reoxida-
30 tion of "scrambled" material seems to yield a more
homogeneous preparation of r31 kD, but the concentration

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of thioredoxin which had to be used was about 100 μ M.
"Scrambled material" is r31 kD protein which is apparently improperly folded due to the formation of one or more incorrect disulfide bonds.

3. Purification

3.1 Phenyl-Sepharose chromatography: The large volume of refolded r31 kD is first centrifuged to remove the insoluble pellet which contains either "scrambled" r31 kD or contaminants. The supernatant is brought to 0.2 M ammonium sulfate in Buffer A and loaded onto a phenyl-Sepharose column equilibrated with Buffer A containing the same ammonium sulfate concentration. The r31 kD protein is then purified by lowering the salt concentration, i.e., by elution in Buffer A.

Example: After reoxidation of the crude protein mixture, extracted from a 10 gram pellet, the suspension of refolded and "scrambled" r31 kD, as well as insoluble contaminants, is subjected to centrifugation at 13,000 rpm (17,000 x g) in a high-speed Beckman centrifuge equipped with a J-14 rotor. The supernatant (1,280 ml) was brought to 0.2 M in ammonium sulfate (AS) and loaded onto a 45 ml column of phenyl-Sepharose previously equilibrated with Buffer A containing 0.2 M AS. The column was washed with 150 ml of the same solution, followed by 150 ml of Buffer A, 50 ml of water and 50 ml of 6 M GuCl (Figures 51 and 52).

5 3.2 Heparin-Sepharose and ion-exchange chromatographies: The
final step of purification of the r31 kD fragment is
chromatography on Q-Sepharose from which it elutes in the
flow-through fraction or on Heparin-Sepharose from which
it is eluted by using a salt gradient. It also binds to
10 S-Sepharose, but the eluted material is still contaminated with most of the impurities.

Example: Approximately 1/2 of the Buffer A peak was
concentrated and purified on a 10 ml Heparin-Sepharose
15 column, from which it was eluted by a solution of 0.5 M
NaCl in Buffer A (Figure 53). The concentrated 31 kD was
dialyzed against Buffer A, pH 8.5, before being loaded
on a 40 ml column of Q-Sepharose, which had previously
been equilibrated with the same buffer. The purified
20 r231 kD fragments, which eluted in the flow-through and
wash fractions were concentrated by lyophilization,
before being characterized. The column was washed free
of the contaminant proteins by a step of 1 M NaCl (Figure
54). The purified material is greater than 95% pure
25 (Figure 51).

3.3 Re-extraction of the pellet: After the refolding
procedure the pellet was re-extracted and treated as
above, since it still contained more than 50% of the r31
30 kD fragment probably in "scrambled" form. The total
yield (including the re-extraction step) of the process,
after 3 columns, was about 10% (Table VII).

5 3.4 Characterization: The r31 kD protein has been charac-
terized and compared to its plasma derived counterpart
in terms of its purity (purity profile on reduced gels
of SDS-PAGE), migration position in non-reduced gels of
10 SDS-PAGE (Figure 51), apparent molecular weight (approx-
imately 37 kD) on Superose 12 (Figure 55), immunoblot and
behavior on Heparin-Sepharose (the NaCl concentration for
elution of both materials from Heparin-Sepharose was
found to be approximately 0.32 M). In all of these
assays the r31 kD protein is similar to plasma derived
15 fibrin binding domain.

4. Comparison between various forms of the r31 kD in terms
of their reoxidation/refolding

20 Three different forms of the r31 kD protein have been defined:

Form a: The protein as it is obtained from the washed pellet,
after dissolution in 6 M GuCl, i.e., in "scrambled" form.

25 Form b: The fully reduced protein, present after treatment
with a reducing agent such as GSH in the presence of 6 M GuCl.

Form c: The reoxidized-refolded protein, obtained by treat-
ment with the GSH/GSSG as described above.

30 These three forms can be distinguished from one another by:

- 5 (i) Their migration on SDS-PAGE gels in the absence of reducing reagents (i.e., without ME), and in the presence of the thiol-trapping agent iodoacetamide (Figure 51); and
- 10 (ii) Their reaction with anti-plasmatc 31 kD on immunoblots of gels run in the absence of ME. Only the correctly reoxidized-refolded form reacted with the antibody.

15 The work described above on reoxidation in the presence of GSH/GSSG (or in the presence of cysteine/cystine) has shown that the r31 kD protein refolds with time to form (c) which is indistinguishable from that of the plasma derived fibrin binding domain.

Characterization of the "scrambled" r31 kD protein:

20 The protein seems to be less soluble in this form, as evidenced from the large amounts remaining in the pellet after extraction.

25 Also the scrambled protein differs from the refolded protein in its binding characteristics to both phenyl-Sepharose and Q-Sepharose.

Characterization of the fully reduced r31 kD protein: This
30 protein form is both more soluble and more homogeneous than the "scrambled" one. After solubilization in 6 M GuCl in the presence of GSH (or DTT or cysteine), and then lowering the

concentration of denaturant to almost zero, the "reduced"
5 protein can be purified on phenyl-Sepharose in the presence
of GSH. Finally the protein is "reoxidized" with GSSG at 1/10
of the concentration of GSH, i.e., 0.3 mM. This did not yield
refolded 31 kD, but a form of "scrambled" protein different
10 from that described in the previous paragraph, probably
because the reduced protein slowly autooxidizes to a scrambled
form, even before it is exposed to GSSG.

5. Preparation of reduced-carboxamidated 31 kD protein

15 Purified plasma derived or recombinant 31 kD protein (ap-
proximately 0.6 mg/ml) were reduced in 4.3 M Guanidinium
Hydrochloride (GuCl), 40 mM β -mercaptoethanol (ME), in 10 mM
Tris-HCl, pH 8.5 for 24 hours at room temperature.

20 Carboxamidation was achieved by adding iodoacetamide to the
protein in four-fold excess over the concentration of ME and
the solution incubated for 1 hour at room temperature.
Subsequently the GuCl concentration was reduced by gradual
dialysis (to 3M, 2M, 1M and 0.5M GuCl), before being dialyzed
25 against Buffer A. The precipitate formed was centrifuged and
the concentration of the resulting 31 kD protein in the
supernatants were 0.34 and 0.19 mg/ml for the plasma derived
and recombinant 31 kD, respectively.

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5 6. Biological activity

10 Anti-platelet aggregation activity: In preliminary experiments with the plasma derived 31 kD, an inhibitory effect had been observed using the platelet aggregation assay (Example 8). Thus far attempts to repeat the experiments demonstrating anti-aggregation properties of the plasma derived 31 kD in the APA have been unsuccessful.

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TABLE VII

Purification of r31kD

	STEP	VOLUME	PROTEIN CONC.	TOTAL PROTEIN	PURITY	AMOUNT 31kD	YIELD	PURIF. DEGREE
		(ml)	(mg/ml)	(mg)	(%)	(mg)	(%)	
5	Extracted pellet			~2000	35	~700	100	1
	Refolding	1280	0.332	425	70	295	42	2.0
	Phenyl-S.	220	0.56	123	85	105	15	2.4
10	"(1/2)			61		52		
	Heparin-S.	16	2.19	35	90	32	9	2.6
	Q-Sepharose	100		23 ^a	>95	22	6	>2.7
	Re-extracted pellet			234			23	
15	Phenyl-S.			96			14	

% Purity determined from SDS-PAGE gels (+ME)

^a Measured after lyophilization.

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EXAMPLE 25

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Pharmacodynamics of the amino terminal 31 kD-fragment of human
plasmatic fibronectin (31 kD) in rats

10 In order to elucidate the metabolic behavior of the amino-terminal fragment of FN, a purified preparation of the tryptic 31 kD fragment of human plasma derived FN (prepared as described in Example 8) was iodinated with ^{125}I by the IC1 method (Vogel et al., PNAS 69: 180-184 (1972)) and injected intravenously into 9 rats (4.5×10^6 cpm; 0.5 mg/Kg). Blood
15 samples were withdrawn 10 minutes after injection and then at 1, 4, 7 and 24 hours after injection. Groups of 3 rats each were sacrificed at 1.7 and 24 hours, and a variety of organs were excised and analyzed for radioactivity. The 24 hour group rats were kept in individual metabolic cages, and
20 accumulated urine and feces were collected at 7 hours and 24 hours. In order to determine whether the radioactivity detected represents the intact protein or small degraded fragments, the samples were subjected to TCA precipitation.

25 The pharmacokinetics of total and TCA-precipitated radioactivity in serum are presented in Figure 56. As shown, the blood levels of the injected 31 kD protein (TCA-precipitable) dropped sharply to about 30% of the initial value during the first hour. The extent of degradation at that point was about
30 40%. Blood levels further declined during the subsequent 3 hours to about 10% of the initial value. A first-order line fitted to the first 3 points of TCA-precipitable values

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(10 minutes, 1 hour and 4 hours) was consistent with a half-life of 1.5 hours. The subsequent decrease in the TCA precipitable cpm in the serum was at a markedly lower rate ($t_{1/2}$ =11 hours), while the extent of degradation did not increase (about 50-70% TCA-soluble radioactivity in the serum).

10 In the urine, 30% of the injected radioactivity was excreted during the first 7 hours, and the rest (>90%) was excreted after 24 hours.

15 All the urinary radioactivity was TCA soluble. The analysis of a variety of organs (kidney, stomach, liver, lung, uterus, ovary, adrenal, colon, ileum, skin, brain, eye, muscle, bladder, heart, spleen, trachea, aorta and vena-cava) did not reveal any specific accumulation and the kinetics of disappearance of the radioactivity followed a pattern similar to
20 that of the blood. In most of the organs, the specific radioactivity (cpm/gr. tissue) was lower than that of the serum; the exceptions were the ileum and stomach at 7 hours, which had four-fold higher value. TCA analysis of the
25 homogenate of stomach and ileum tissue at 7 hours revealed that most of the label was associated with small degradation products of the protein.

The results indicate that exogenous plasma derived 31 kD
30 amino-terminal fragment of FN is moderately degraded and excreted in the body. The pharmacokinetic behavior is not consistent with a first-order kinetics, which may indicate

that the protein is moderately distributed in the tissues and
5 body compartments other than blood. This is also evident from
the finding that the degree of degradation does not increase
during the 4-24 hour period, thus reflecting a gradual release
of the protein from body compartments. The exclusive and
relatively early appearance of the metabolites in the urine
10 indicates that the protein is readily excreted through the
kidneys. The lack of accumulation of the material in the
liver may be an indication that this organ is not a major
locus of degradation and is not involved in detoxification.

15 The relatively short half-life of the 31 kD FBD is important
for its possible use in diagnostic imaging of thrombi. The
plasma derived 31 kD FBD or the recombinant 31 kD FBD (r31
kD) may be labeled radioactively or by other means and then
introduced into the blood for the purpose of imaging thrombi.

20 The shorter half-life of the molecule is also important when
utilizing it to prevent clot formation. By contrast, Heparin,
the current therapeutic agent of choice, suffers from a very
long half-life.

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EXAMPLE 26

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Biological activity of the recombinant Fibrin Binding Domain (r31 kD)

The biological activity of the purified recombinant 31 kD FBD protein was compared to biological activity of either human plasma derived FN or a 31 kD FBD protein derived from partial tryptic digest (Example 8) of human plasma derived FN. The biological activities assayed were binding to fibrin clot in vivo and in vitro, binding to bacteria (Staphylococcus aureus) and binding to extracellular matrix.

15

I. Fibrin binding

Two sets of experiments were carried out. In the first set, the binding of ^{125}I -r31 kD to fibrin was monitored during clot formation (Reaction I), while in the second set of experiments, the binding of ^{125}I -r31 kD to fibrin was monitored at various time periods after clot formation (Reaction II). Thrombin or Ca^{++} were added to the mixtures in order to enable clot formation in citrated blood.

25

Materials and MethodsFibronectin Fibrin Binding Domain: FBD

30

A. Binding of FBD to fibrin:

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Plasma derived 31 kD FBD was derived by partial tryptic digest
5 of human fibronectin (see Example 8).

Production of r31 kD FBD in Escherichia coli and its subse-
quent purification from the pellet of cell lysate was per-
formed according to Example 24. Fibronectin was obtained from
10 human plasma. ^{125}I -labeling of FBD and FN was carried out by
the IC1 method (31). The labeled proteins having specific
activity of 20-200 cpm/ng, were stored at -20°C in small
aliquots in a solution of 0.1% BSA-PBS and used within 2
weeks.

15

1. Binding of ^{125}I -FBD to fibrin clot during its formation
(Reaction I):

The complete reaction mixture in siliconized microfuge tubes
20 contained in a final volume of 250 μl the following com-
ponents:

20-200 μl human whole blood (fresh, non-citrated, or 1-
7 days old, citrated, as indicated in the figure legends)

25

0.1% BSA
5 mM CaCl_2
1U/ml Thrombin
 ^{125}I -r31 kD FBD

30

When binding was measured using non-citrated blood, CaCl_2 and
thrombin was not added ("Naive Thrombii").

35

5 All ingredients were prepared in PBS. The reaction was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of EDTA (25 mM), and centrifugation in a microfuge centrifuge at maximum speed for 3 minutes. The supernatant was discarded and the pellet was washed twice in
10 1 ml PBS, 0.1% BSA, 5 mM EDTA, 1 mM PMSF. The radioactivity in the pellet was monitored by a gamma counter.

When competition with non-radioactive protein was carried out, the competing protein was added together with the ^{125}I -labeled
15 protein at the concentrations indicated in the figure legends.

2. Binding of ^{125}I -r31 kD FBD to preformed fibrin clots (Reaction II):

20 The reaction mixture contains the same components as indicated for Reaction I, except for the ^{125}I -r31 kD. The first incubation was carried out at 37°C for 30 minutes, and only then the ^{125}I -r31 kD was added and the reaction was further incubated for a second period of 30 minutes. Reaction II was terminated
25 and measured as described above for Reaction I.

Results

30 A. Binding of ^{125}I -r31 kD to fibrin: effect of thrombin and Ca^{++}

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5 The effect of thrombin and Ca^{++} on ^{125}I -r31 kD binding to fibrin during clot formation (Reaction I) or to preformed clots (Reaction II) was studied in citrated human whole blood (Figure 57).

10 Hirudin, a specific inhibitor of thrombin (32) reduced the binding of ^{125}I -r31 kD to the fibrin clot in Reaction I, indicating that thrombin is needed for the binding. When thrombin is inhibited there is a reduction in clot formation and less fibrin is available for binding. The addition of
15 citrate to blood significantly reduces the concentration of free Ca^{++} in the serum and therefore the addition of Ca^{++} for fibrin clot formation is obligatory. However, binding of r31 kD to a preformed clot is reduced when carried out in serum which has been already depleted of free Ca^{++} ions by the preformed clot. Addition of Ca^{++} to the Ca^{++} depleted serum
20 increases the r31 kD binding. This effect of Ca^{++} on binding was dramatically demonstrated when binding was measured in PBS. The addition of Ca^{++} increased the binding, even to a higher extent than observed in reaction I. Thus, both clot formation and r31 kD binding are Ca^{++} ion dependent.

25 Thus, binding of ^{125}I -r31 kD to fibrin in Reaction II increases when the assay is carried out in PBS (Phosphate Buffer Saline solution) instead of serum.

30 B. Release of ^{125}I -r31 kD from fibrin clot by Plasmin

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In order to determine whether the ^{125}I -r31 kD is covalently bound to the fibrin in the clot, plasmin, which is known (33,34) to cleave the N-terminal domain of plasma derived FN between amino acids Arg²⁵⁹ and Thr²⁶⁰ was added to the clot after ^{125}I -r31 kD was bound. The incubation with plasmin was carried out for various time intervals (Figure 58).

The results demonstrate that plasmin caused a reduction in the radioactivity bound to the clot (pellet). Reduction of radioactivity in the pellet was time dependent and could be attributed to the fact that plasmin cleaved the r31 kD FBD. The amount of radioactivity monitored in the supernatant increased with time. Upon loading of the plasmin soluble reaction fraction (the supernatant) on SDS polyacrylamide gels it is possible to detect the shortened form of the cleaved ^{125}I -r31 kD. The release of ^{125}I -r31 kD from the fibrin clot occurs only by plasmin cleavage and not by heating with a solution containing SDS, EDTA and β -mercaptoethanol to 100°C, thus demonstrating that ^{125}I -r31 kD is covalently bound to the fibrin clot.

3. Binding of ^{125}I -r31 kD to fibrin during clot formation: effect of unlabeled r31 kD and other molecules

The ability of various cold recombinant and plasma derived FBD preparations as well as human plasma derived fibronectins at different concentrations to interfere with the binding of ^{125}I -rFBD to fibrin during clot formation (Reaction I) was studied (Figure 59). The results obtained demonstrate that

the binding of $0.15 \mu\text{M}$ ^{125}I -rFBD to fibrin was similar or even increased in the presence of unlabeled r31 kD, unlabeled plasma derived 31 kD or unlabeled FM at concentrations up to 20 folds excess ($3 \mu\text{M}$).

However, when the amount of newly formed fibrin clot was reduced, using either suboptimal concentrations of CaCl_2 and thrombin (1 mM and 0.3 units/ml , respectively), or by reducing the volume of the blood in the reaction to $1/10$ of the amount originally specified, the competition between the unlabeled FBD and the binding of ^{125}I -FBD for binding to the clot became significant.

In order to further study the specificity of the FBD binding reaction, we compared the binding of r31 kD to the binding of its reduced forms (Example 24).

For these studies we used one batch of reduced-carboxamidated plasma derived 31 kD, one batch of reduced-carboxamidated r31 kD and one batch of fully-reduced r31 kD all prepared as described in Example 24. Surprisingly, these various reduced forms of 31 kD FBD caused a dramatic reduction in the binding of the ^{125}I -FBD (reduction of 40-80% by $0.3\text{-}3.0 \mu\text{M}$; Figure 59). We have also noticed a dramatic decrease in the size of the newly formed clot (Reaction I) in the presence of the fully reduced FBD. Clot formation was totally inhibited in the presence of high concentrations of the fully reduced FBD (above $5 \mu\text{M}$).

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Since a similar inhibitory effect was exhibited by the various reduced forms of the 31 kD proteins on the binding of refolded ^{125}I -FBD to a preformed fibrin clot (Reaction II), the possibility of interference with the crosslinking reaction of the FBD to the fibrin clot catalyzed by the serum transglutaminase factor XIIIa, was suggested.

4. Binding of ^{125}I -FBD to the fibrin thrombi (Reaction II): effect of transglutaminase inhibitors

Transglutaminases are a class of calcium ion-dependent enzymes that catalyze an amidation reaction in which a carboxamide group of peptide bound glutamyl residues and primary amines, including the epsilon amino group of peptide bound lysyl residues, are crosslinked. Plasma FN is a substrate for transglutaminase from plasma, factor XIIIa, (thrombin activated blood coagulation factor XIII) or liver; FN can be crosslinked to itself, fibrin and collagen.

The glutamyl residues which are susceptible to factor XIII crosslinking are localized in the FBD region of FN (35).

The binding of ^{125}I -FBD to preformed fibrin clot (Reaction II) in the presence of various concentrations of the primary amines spermidine and putrescine, the classical transglutaminase inhibitors, was studied (Figure 60). The reaction was 50% inhibited by about 5 mM spermidine or putrescine. In parallel to the expected inhibition by the primary amines, a dramatic inhibition of the binding was also observed by the

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reduced-carboxamidated FBD, with a half maximal reduction at around 2.5 μ M.

5

5. Binding of ^{125} I-r31 kD to preformed fibrin clot: effect of aging on binding

10 For imaging purposes, it was important to determine the effect of clot aging on FBD binding capacity.

The binding of ^{125} I-r31 kD to preformed fibrin clot was determined. ^{125} I-r31 kD was added to the preformed clots (1, 4 or 24 hours) and binding to fibrin was monitored (Figure 61).

15

The binding of ^{125} I-r31 kD to the fibrin clot was higher after 24 hours than the binding after 1 or 4 hours, presumably due to the fact that the clot formed became larger with time.

20

As indicated in experiment I of this example, the presence of free Ca^{++} ions in citrated blood is important in order to obtain optimal binding and crosslinking of ^{125} I-r31 kD to the fibrin clot. Thus, in serum where Ca^{++} was already depleted by the preformed clot the binding of ^{125} I-r31 kD to the fibrin clot was low while in the PBS containing 5 mM CaCl_2 , the binding was high at all incubation times.

25

30 When 300 μ g/ml of plasma derived FN was added as competitor in the presence of Ca^{++} , there was a 20% reduction in ^{125} I-

35

5 r31 kD binding to the fibrin clot probably due to some competition on the available fibrin binding sites.

6. Binding of ^{125}I -r31 kD to "Naive" thrombi (Reactions I and II): effect of thrombi age on the binding

10 The ability to differentiate between "old" (preformed) and "newly formed" thrombi, is an important requirement of a probe for thrombus imaging. Figure 62 shows experiments designed to compare ^{125}I -r31 kD binding to "old" and "newly formed" clots. In the first experiment ^{125}I -r31 kD was added at the
15 same time as the initiation of clot formation ("newly formed") and was allowed to interact with the clot during seven days of incubation. It is evident that FBD is incorporated efficiently and the amount incorporated increased during the first two days, probably representing an increase in the clot
20 size during that period. In the second experiment ^{125}I -r31 kD was added to aged clots (1-7 days old) for a limited period of 2 hours ("preformed"). The extent of FBD incorporation remained constant, regardless of the clot age. Note, however, that binding in this protocol is lower than in that of the
25 first experiment. These experiments suggest that the probe ^{125}I -r31 kD may be used in two different protocols to differentiate between "old" clots - thrombi and those which are still in the process of growing.

30 7. Plasma "Thrombin Time" ("TT")

The effect of the r31 kD protein on clotting of whole blood was measured using the clinical laboratory parameter defined as Thrombin-Time. In this reaction aliquots of 100 μ l citrated healthy human plasma were mixed with 100 μ l PBS and either the transglutaminase inhibitor spermidine, or with reduced-carboxyamidated p31 kD or reoxidized-refolded r31 kD. 200 μ l thrombin solution was added to each aliquot while continuously mixing. The time from the addition of the thrombin to the formation of clot was measured, and is expressed as "TT" in seconds (Table VIII).

8. Binding of ^{125} I-r31 kD to fibrin (Reaction I): effect of exogenous transglutaminase and "reduced-carboxamidated" FBD

In Section 4 of this example we demonstrated that transglutaminase inhibitors reduce the binding of ^{125} I-r31 kD to clots. In this section we studied the effects of exogenous transglutaminase from pig liver on the binding reaction.

The results presented in Figure 63 demonstrate that the addition of the exogenous transglutaminase dramatically increases the binding of ^{125} I-r31 kD to clots, indicating that this enzyme may be a rate limiting step in this reaction.

Moreover, both endogenous and exogenous transglutaminase dependent binding activities were equally decreased by the reduced-carboxamidated FBD (approximately 56% and 72% inhibition by 0.3 μ M and 1.5 μ M "reduced" FBD, respectively).

5 These results strongly indicate that the "reduced" forms of FBD inhibit the binding of the refolded form of FBD to the fibrin clot by interfering with the transglutamination and cross-linking reaction. This probably causes destabilization of the fibrin clot.

10 9. Binding of ^{125}I -FBD to Extra Cellular Matrix (ECM)

Adhesive molecules such as von Willebrand factor, fibronectin, fibrinogen, thrombospondin, collagen and laminin bind to ECM
15 formed by removal of endothelial cells. r31 kD FBD can serve for imaging the initial steps in plaque formation at the site of injury. The binding of ^{125}I -r31 kD at various concentrations to ECM was studied in the presence or absence of thrombin. The results demonstrated that the binding of ^{125}I -
20 r31 kD at low concentrations to ECM was not affected by thrombin ($0.3\ \mu\text{M}$). At higher concentrations of the thrombin binding of r31 kD was slightly higher indicating that the number of binding sites naturally present are limited and thrombin digestion might expose additional binding sites
25 (Figure 64).

The fact that ^{125}I -r31 kD can bind to ECM indicates that it might be useful for imaging the initial plaque formation in the denudated blood vessel.

30

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TABLE VIII

THROMBIN TIME ("TT")

5

Effect of FBD ("reduced" and "folded") and Related Molecules

10	Thrombin Conc. (μ /ml)	Additions	"TT" Values		
			(Seconds)	(%)	(\)
I	2.5	-	25.5	100	-
		1mM Spermidin	44	172	72
		1 μ M "R" FBD	34	133	33
		2.5 μ M "R" FBD	39	152	52
		2.5 μ M "F" FBD	26.5	103	03
20 ^{II}	1.25	-	55.4	100	-
		1mM Spermidine	114	205	105
		2.5 μ M "R" FBD	81	146	46
		2.5 μ M "F" FBD	58	104	04

25

"R" FBD = Reduced-carboxamidated p31kD.

"F" FBD = Refolded r31kD.

30

35

II. Bacteria Binding

5 The involvement of fibronectin in adhesion and invasion of
wounds by a wide range of gram-positive bacteria is well
established (36). The fibrin binding domain of authentic
plasma derived FN has been shown to interact with high
10 affinity to specific receptors on the surface of bacteria.
The sites at which Staphylococcus aureus typically initiates
infection are rich in FN, e.g. blood clots and subendothelium.
Furthermore, exogenous FN enhances bacterial adhesion to these
sites. FN binds to S. aureus through saturable, specific
15 surface protein receptors. Scatchard analysis has revealed
high affinity receptors with binding constant of $5 \times 10^{-9} \text{M}$, and
a range of 100-20,000 receptors per bacterium (37). The
expression of FN receptors correlates with invasiveness and
pathogenicity of the clinical isolates. Removal of the
20 receptors from S. aureus by mechanical means, or by growth of
the bacteria in the presence of antibiotics decreases their
ability to adhere to FN. As FN is a divalent molecule
consisting of multiple functional domains with cell binding
and collagen binding activities in addition to bacterial
25 binding, it can anchor the bacteria to the wound via the
various components of the extracellular matrix as well as via
the FN receptor in tissue cells.

30

35

Materials and Methods

5

Binding of Bacteria to labeled FN or FBD in solution

A. Direct binding reaction

10 Various concentrations of ^{125}I -r31 kD FBD or ^{125}I -FN, were added to 5×10^8 S. aureus bacteria in a PBS solution additionally containing 0.1% Tween and 1% BSA. The final volume was 1 ml. Total radioactivity in the reaction was assayed using a 20 μl aliquot taken immediately after the addition of the
15 bacteria.

The mixture was incubated for 2 hours at 20°C.

The amount of binding was assayed by removing 100 μl of the
20 incubation mixture and layering on top of 0.5 ml PBS layered on 3 ml 10% percol, 0.15 M NaCl in a 5 ml siliconized tube. This was then centrifuged at 1,350 x g (4,000 rpm in SW bucket rotor) for 15 minutes at 20°C. The supernatant was aspirated and the pellet assayed for radioactivity.

25

B. Competition with unlabeled FN, FBD and related molecules

The procedure followed was identical to the above procedure except that 3 $\mu\text{g/ml}$ ^{125}I -p31 kD was used and the specified
30 amounts of the competing molecule (FN or FBD) was also added to the initial binding mixture.

35

II. Binding of radioactively labeled bacteria to immobilized
5 FN

Plastic vials were coated with 0.3 ml of 50 μ g/ml FN, or 1% BSA.

10 The tubes were incubated with shaking at 4°C overnight. The tubes were then washed with 5 ml PBS three times. Then 0.3 ml of 1% BSA in PBS was added and the tubes were further incubated with shaking for 2-3 hours at 20°C (for blocking free sites).

15 In indirect-binding experiments, the bacteria were pre-incubated with inhibitor, at 4°C for 2 hours.

The bacteria (4×10^6 pfu/ml, 3 pfu/cpm) were added to the vials
20 at concentrations indicated in the figure legends. The final volume of the assay mix was 0.3 ml PBS. The mix was slowly agitated at 4°C for 90-120 minutes.

The tubes were then decanted and washed with 5 ml PBS three
25 times.

5 ml of scintillation-liquid was added when assaying for binding of ^3H -labeled bacteria.

30 III. Binding of labeled bacteria to catheters

5 Catheters ["UNO" sterile bronchial plastic catheters (18 CH size; Unoplast A/S, Denmark)], were cut to 1 cm and 2 cm pieces, weighed and then cut once lengthwise.

10 The catheter pieces were incubated with 50 μ g/ml FN at 4°C overnight with shaking. The controls were incubated with PBS under the same conditions.

15 The FN solution was then decanted and the catheters were washed three times in PBS. BSA-blocking was performed by adding 1% BSA in PBS for 1-2 hours at 20°C. The catheters were again washed three times with PBS.

20 Bacterial binding was performed as described in the previous section except that the catheter pieces were added to the assay mixture. The final volume used was 3 ml. The reaction was performed in 10 ml tubes. The reaction was incubated for 2 hours at 20°C. For the binding assay ^{125} I-S. aureus (4×10^6 pfu/ml, 3 pfu/cpm) were used. (When H³-leucine-labeled S. aureus was used the specific activity was 1 cpm/ 3.3×10^3 pfu. Labeling was performed according to P.B. Russel et al. (38).
25 When ^{125} I-labeled S. aureus was used the specific activity was 1 cpm/3 pfu. Labeling was performed according to A.E. Bolton and W.M. Hunter (39).) The competing molecules at the designated concentrations were preincubated with the bacteria for 30 minutes at 20°C. The catheters were then washed three
30 times with PBS and then directly counted in a gamma counter.

Results

5 A. Binding of bacteria to ^{125}I -FN or FBD in solution

I. Direct Binding

10 Experiments were performed in order to determine the binding of ^{125}I -FN or ^{125}I -rFBD to S. aureus bacteria in suspension. Various amounts of radioactive FN or r31 kD were added to 5×10^8 bacteria incubated for 2 hours and then centrifuged over a 10% Percoll-saline solution. Radioactivity was monitored in the pellet (Figure 65).

15 The results showed increased binding of ^{125}I -rFBD (r31 kD) to the bacteria in suspension as compared to the binding of the ^{125}I -FN.

20 This increased binding of ^{125}I -rFBD to S. aureus as compared to ^{125}I -FN binding to S. aureus can be attributed to a higher affinity of a monovalent domain in comparison to bivalent multidomain of intact plasma derived FN.

25 II. Binding of ^{125}I -plasma derived 31 kD FBD (p31 kD) to S. aureus: competition with "native" unlabeled FN, FBD and related molecules

30 A fixed amount of ^{125}I -p31 kD ($3 \mu\text{g/ml}$) was incubated with 5×10^8 bacteria in the presence of increasing amounts of various FBD molecules as competitors (Figure 66).

5 The results demonstrate that "native" FN, p31 kD or rFBD inhibited the binding of ^{125}I -p31 kD to S. aureus in a similar fashion, indicating that rFBD is as active as the natural plasma derived molecules. However, the reduced forms of recombinant or plasma derived FBD only minimally inhibit the binding of ^{125}I -FBD to the bacteria, indicating, that proper
10 folding is necessary for binding. A related recombinant protein (33 kD CBD of FN) which does not have a bacterial binding site did not inhibit ^{125}I -pFBD binding to S. aureus.

15 B. Binding of labeled S. aureus to immobilized FN

To estimate the capacity of rFBD (r31 kD) to interfere with the adherence of bacteria to the extracellular matrix in wounds, a competition assay was developed. In this assay, adherence of S. aureus to plastic surface coated with FN, and
20 the interference of FBD with the binding was measured (see Figure 67).

The results demonstrate that the adhesion of S. aureus to FN coated plastic vials was inhibited following pre-incubation
25 of S. aureus with FN, pFBD or rFBD. The extent of inhibition by these molecules was similar. A non-related protein, BSA, which does not have S. aureus binding sites, did not cause any inhibition in adhesion of radioactive labeled S. aureus to FN coated plastic vials.

30

C. Binding of S. aureus to bronchial catheters: effect of FBD and heparin

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5 Catheter sepsis due to various species of S. aureus contributes to the high incidences of serious clinical complications.

10 We have examined the ability of S. aureus to bind FN coated catheters. Figure 68 demonstrates that the binding of S. aureus to the FN coated catheters is quite high, approximately 10^4 PFU/cm².

15 Preincubation of bacteria with increasing concentrations of r31 kD reduced the binding of the bacteria to the catheters. The IC₅₀ for this inhibition is between 0.08 - 0.8 μ M (Figure 68). Similar inhibition was also obtained with r20 kD FBD and p31 kD FBD.

20 Systemic administration of heparin and/or the use of heparin bonded polyurethane catheters is reported to decrease the incidence of thrombosis.

25 Therefore we also measured the inhibitory effect of FBD on S. aureus attachment to the catheters in the presence of 5 μ M heparin. The results (Figure 68) demonstrate that heparin did not affect the binding of the bacteria to catheters, however, r-FBD inhibition of bacterial binding remains constant even in the presence of heparin. This indicates the utility of r31
30 kD for inhibition of S. aureus colonization and sepsis in a clinical setting, even in the presence of heparin.

Conclusion

5 These results demonstrate that the r31 kD FBD or the plasma
derived 31 kD FBD may be used therapeutically in preventing
bacterial colonization of wounds. The various FBD proteins
will be formulated in suitable pharmaceutical formulations
10 well-known to the average man of the art, and then used to
"irrigate" or "flood" or treat the wound area for a suitable
period of time, thereby preventing bacterial colonization of
the wound.

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EXAMPLE 27

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Adhesion of plasma derived 31 kD FBD to thrombi in a rabbit aorta lesion model

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In order to demonstrate the ability of the fibrin binding domain to adhere to thrombi in vivo, a model of thrombus formation in rabbits was used. In this model, the endothelial layer of a segment of the aortal wall is removed with a balloon catheter, thus exposing the lamina intima. This results in the subsequent formation of a film of thrombi in the lesion area. It has been demonstrated by Uehara et al. (40) that labeled FN given systemically to rabbits exhibited extensive binding to such thrombi, as indicated by a higher specific radioactivity in the lesion part of the aorta as compared to adjacent untreated segments.

20

25

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In order to compare the plasma derived 31 kD FBD fragment with intact plasma-derived FN, the two molecules were iodinated with ^{125}I using the ICL method. Two hours after de-endothelialization, the radio-labeled molecules were injected intravenously into rabbits (20 μCi ; 100 micrograms/kg; 3 rabbits per group). At 72 hours after injection, the aortas were removed, the scraped (abdominal) and intact (thoracic) areas were separated and each part was cut into several segments (5-6 segments for the lesion part and 2 segments for the control part). The tissue pieces were weighed and radioactivity counted.

35



5 Figure 69 summarizes the specific activity values found in the sequential aorta slices in rabbits injected with ^{125}I -FN (rabbits No. 1-3) or ^{125}I -p31 kD FBD (rabbits 4-6). As can be seen in Figure 69, enhanced localization of the labeled molecules was found in the de-endothelialized segments (thrombus zone). Also more radioactivity was localized in the
10 clots when labeled p31 kD FBD was used than when labeled FN was used.

15 These results demonstrate that molecules comprising the FBD moiety should be useful for the imaging of arterial thrombus clots.

When used for imaging it may be desirable to attach to the FBD (plasma derived or recombinant) other markers useful in imaging such as enzymes or radio-opaque inert molecules.
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EXAMPLE 28

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Effect of the 33 kD cell binding domain on platelet aggregation

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Additional studies were performed to analyze the effect of the recombinant 33 kD cell binding domain on platelet aggregation. Except as specified, the experiments were performed as in Example 7.

15

Aggregations in platelet rich plasma (PRP) or whole blood (blood) were induced by ADP (10 μ M). Extent of aggregation in PRP was determined by light transmittance. Extent of aggregation in whole blood was measured by the impedance technique. The recombinant human fibronectin cell binding domain (33 kD) or the pentapeptide GRGDS were added to the reaction mixture to the final concentrations indicated in the table.

20

The results shown in Table IX demonstrate that the recombinant 33 kD CBD is a potent inhibitor for the aggregation of platelets of humans and primates (e.g., baboons).

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TABLE IX

RESPONSE OF PLATELETS FROM VARIOUS SPECIES
TO FIBRONECTIN DERIVATIVES 33kD AND GRGDS

5	PLATELET PREPARATION	FN DERIVATIVE	CONCENTRATION (uM)	INHIBITION (%)
	PRP	33kD	7-15	50
		GRGDS	115	50
	HUMAN			
	blood	33kD	1	50
10		GRGDS	40	50
	BABOON	PRP	33kD	5
		GRGDS	125	50
				50
15		PRP	33kD	30
		"	60	0
	DOG	GRGDS	200	30
				55
	blood	33kD	2	0
		"	6	50
		"	12	50-70*
20	PIG	PRP	33kD	60
		GRGDS	200	0
				5

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References

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

1. A plasmid for expression of a polypeptide which comprises a substantial portion of the amino acid sequence of the N-terminal fibrin binding domain of naturally-occurring human fibronectin, having a molecular weight from about 20 kD to less than about 31 kD, having the amino acid sequence gln-ala-gln-gln or met-gln-ala-gln-gln at the N-terminus of the polypeptide and which does not correspond to a product of proteolytic digestion of naturally-occurring human fibronectin, comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.
2. A cell which comprises the plasmid of claim 1.
3. A bacterial cell according to claim 2.
4. An Escherichia coli cell according to claim 3.
5. A method of producing a polypeptide which comprises a substantial portion of the amino acid sequence of the N-terminal fibrin binding domain of naturally-occurring human fibronectin having a molecular weight from about 20 kD to less than about 31 kD, having the amino acid sequence gln-ala-gln-gln or met-gln-ala-gln-gln at the N-terminus of the polypeptide and which does not correspond to a product of proteolytic digestion of human fibronectin, comprising treating an Escherichia coli cell comprising the plasmid of claim 1 so that the DNA directs expression of the polypeptide and recovering the polypeptide so expressed.
6. A polypeptide which comprises a substantial portion of the amino acid sequence of the N-terminal fibrin binding domain of naturally-occurring human fibronectin having a molecular weight from about 20 kD to less than about 31 kD, having the amino acid sequence gln-ala-gln-gln or met-gln-ala-gln-gln at the N-terminus of the polypeptide and which does not correspond to a product of proteolytic digestion of human fibronectin.
7. A hybrid polypeptide consisting essentially of at least two polypeptides of claim 6.
8. A composition comprising at least two polypeptides of claim 6 or 7 and a suitable carrier.
9. A composition of claim 8, wherein the polypeptides are bound to one another.
10. A pharmaceutical composition comprising an amount of the composition of claim 8 or 9 effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier.
11. A pharmaceutical composition comprising an amount of a polypeptide of claim 6 or 7 effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier.

12. A pharmaceutical composition comprising an amount of a polypeptide of claim 6 or 7 effective to inhibit thromboxane release from platelets and a pharmaceutically acceptable carrier.
13. A use of an amount of a polypeptide of claim 6 or 7, effective to inhibit platelet aggregation, for inhibiting platelet aggregation.
14. A use of an amount of a polypeptide of claim 6 or 7, effective to inhibit thromboxane release from the platelets, for inhibiting thromboxane release from platelets.
15. The use in accordance with claim 13 wherein the platelet aggregation is associated with a cerebrovascular disorder.
16. The use in accordance with claim 13 wherein the platelet aggregation is associated with a cardiovascular disorder.
17. The use in accordance with claim 16, wherein the cardiovascular disorder comprises acute myocardial infarction.
18. The use in accordance with claim 16, wherein the cardiovascular disorder comprises angina.
19. The use in accordance with claim 13 to inhibit platelet aggregation in a subject who has undergone angioplasty, thrombolytic treatment, or coronary bypass surgery.
20. A use of an amount of a polypeptide of claim 6 or 7, effective to promote healing of a wound, to treat a subject with said wound.
21. The use in accordance with claim 20, wherein the wound is a cutaneous wound.
22. The use in accordance with claim 21, wherein the cutaneous wound is an incisional wound, a skin deficit wound, a skin graft wound, or a burn wound.
23. The use in accordance with claim 20, wherein the wound is an eye wound.
24. The use in accordance with claim 23, wherein the eye wound is a corneal epithelial wound or a corneal stromal wound.
25. The use in accordance with claim 20, wherein the wound is a tendon injury.
26. A use of an amount of a polypeptide of claim 6 or 7, effective to prevent or treat bacterial infection, to treat a subject susceptible to, or afflicted with, a bacterial infection.
27. The use of claim 26, wherein the bacterial infection is due to the presence of a catheter or an implant in the subject.
28. A use of an amount of a polypeptide of claim 6 or 7, effective to retard tumor metastasis, to treat a subject with cancer.

29. A method of detecting a tumor in a subject which comprises administering to the subject an amount of a polypeptide of claim 6 or 7 effective to detect the tumor, wherein the polypeptide is labeled with a radioactive or radioopaque label, for detecting the tumor.
30. A method of detecting a thrombus in a subject which comprises administering to the subject an amount of a polypeptide of claim 6 or 7 effective to detect the thrombus, wherein the polypeptide is labeled with a radioactive or radioopaque label, for detecting the thrombus.
31. A polypeptide in accordance with claim 6 or 7 bound to a growth factor.
32. A polypeptide in accordance with claim 31, wherein the growth factor is selected from the group consisting of: epidermal growth factor, platelet derived growth factor, α -transforming growth factor, β -transforming growth factor, fibroblast derived growth factor, tumor necrosis factor, interleukins, interferons, erythropoietin, colony stimulating factor, granulocyte/macrophage-specific colony stimulating factor, granulocyte-specific colony stimulating factor, and colony stimulating factor -I.
33. A polypeptide in accordance with claim 6 or 7 bound to serum albumin.
34. A polypeptide in accordance with claim 6 or 7 bound to a blood factor.
35. A polypeptide in accordance with claim 34, wherein the blood factor is Factor VIII or XIII.
36. A polypeptide in accordance with claim 6 or 7 bound to polyethyleneglycol.
37. A polypeptide in accordance with claim 6 or 7 bound to superoxide dismutase.
38. A medical device comprising the polypeptide of claim 6 or 7 applied as a coating to the surface of the medical device.
39. A medical device of claim 38, wherein the medical device is a catheter.
40. A use of an amount of a polypeptide of any of claim 6 or 7 as a coating for the surface of a medical device effective to minimize risk of bacterial infection associated with use of the medical device.
41. A use in accordance with claim 40, wherein the medical device is a catheter.
42. A use of an amount of a polypeptide of claim 6 or 7, effective to inhibit platelet aggregation, for the production of a medicament for inhibiting platelet aggregation.
43. A use of an amount of a polypeptide of claim 6 or 7, effective to inhibit thromboxane release from the platelets, for the production of a medicament for inhibiting thromboxane release from platelets.
44. A use in accordance with claim 42, wherein the platelet aggregation is associated with a cerebrovascular disorder.
45. A use in accordance with claim 42, wherein the platelet aggregation is associated with a cardiovascular disorder.

46. A use in accordance with claim 45, wherein the cardiovascular disorder comprises acute myocardial infarction.
47. A use in accordance with claim 45, wherein the cardiovascular disorder comprises angina.
48. A use in accordance with claim 42, wherein the platelet aggregation is in a subject who has undergone angioplasty, thrombolytic treatment, or coronary bypass surgery.
49. A use of an amount of a polypeptide of claim 6 or 7, effective to promote healing of a wound, for the production of a medicament for treating a subject with said wound.
50. A use in accordance with claim 49, wherein the wound is a cutaneous wound.
51. A use in accordance with claim 50, wherein the cutaneous wound is an incisional wound, a skin deficit wound, a skin graft wound, or a burn wound.
52. A use in accordance with claim 49, wherein the wound is an eye wound.
53. A use in accordance with claim 52, wherein the eye wound is a corneal epithelial wound or a corneal stromal wound.
54. A use in accordance with claim 49, wherein the wound is a tendon injury.
55. A use of an amount of a polypeptide of claim 6 or 7, effective to prevent or treat bacterial infection, for the production of a medicament for treating bacterial infection.
56. A use of claim 55, wherein the bacterial infection is due to the presence of a catheter or an implant.
57. A use of an amount of a polypeptide of claim 6 or 7, effective to retard tumor metastasis, for the production of a medicament for treating a subject with cancer.

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Figure 1

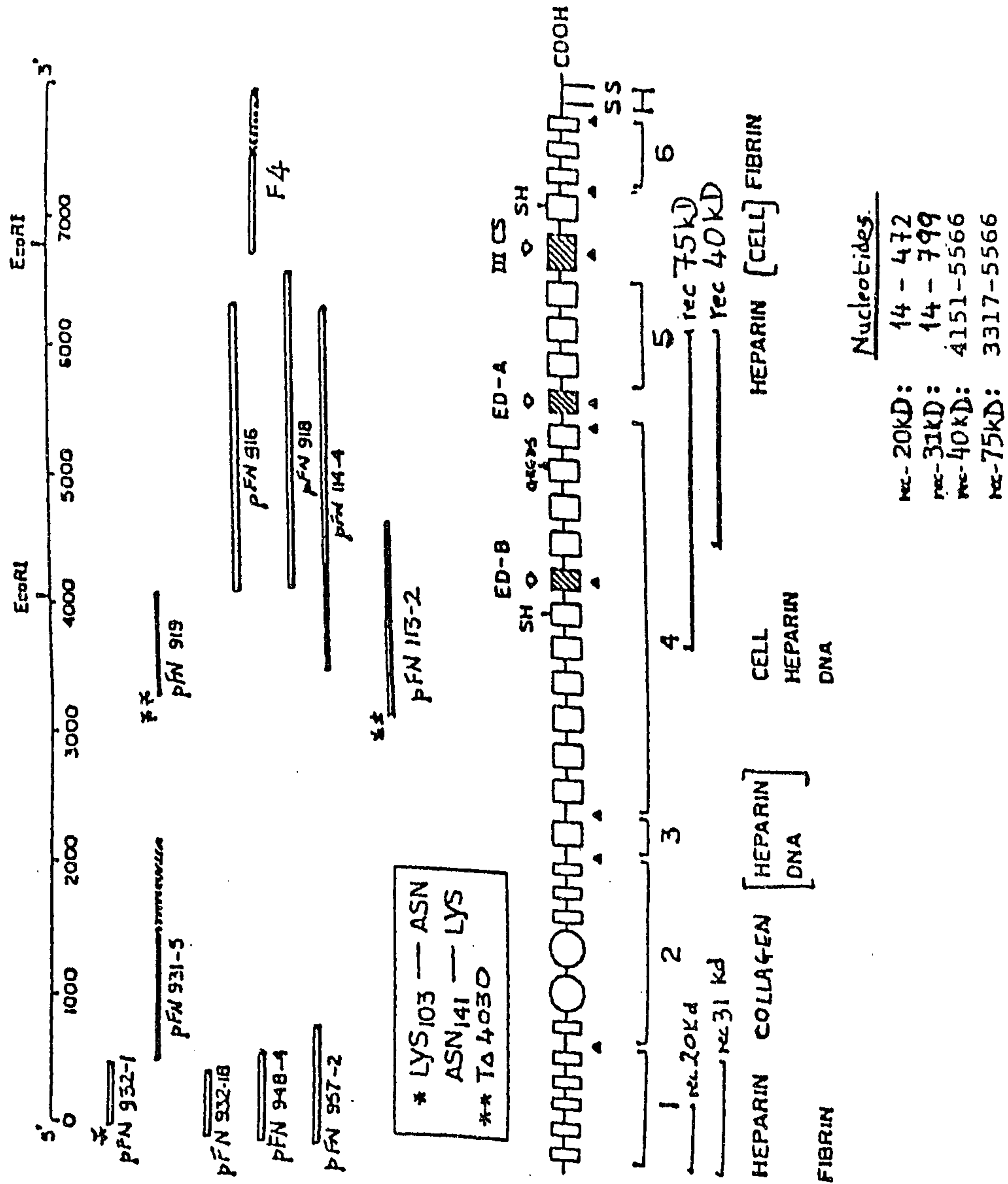
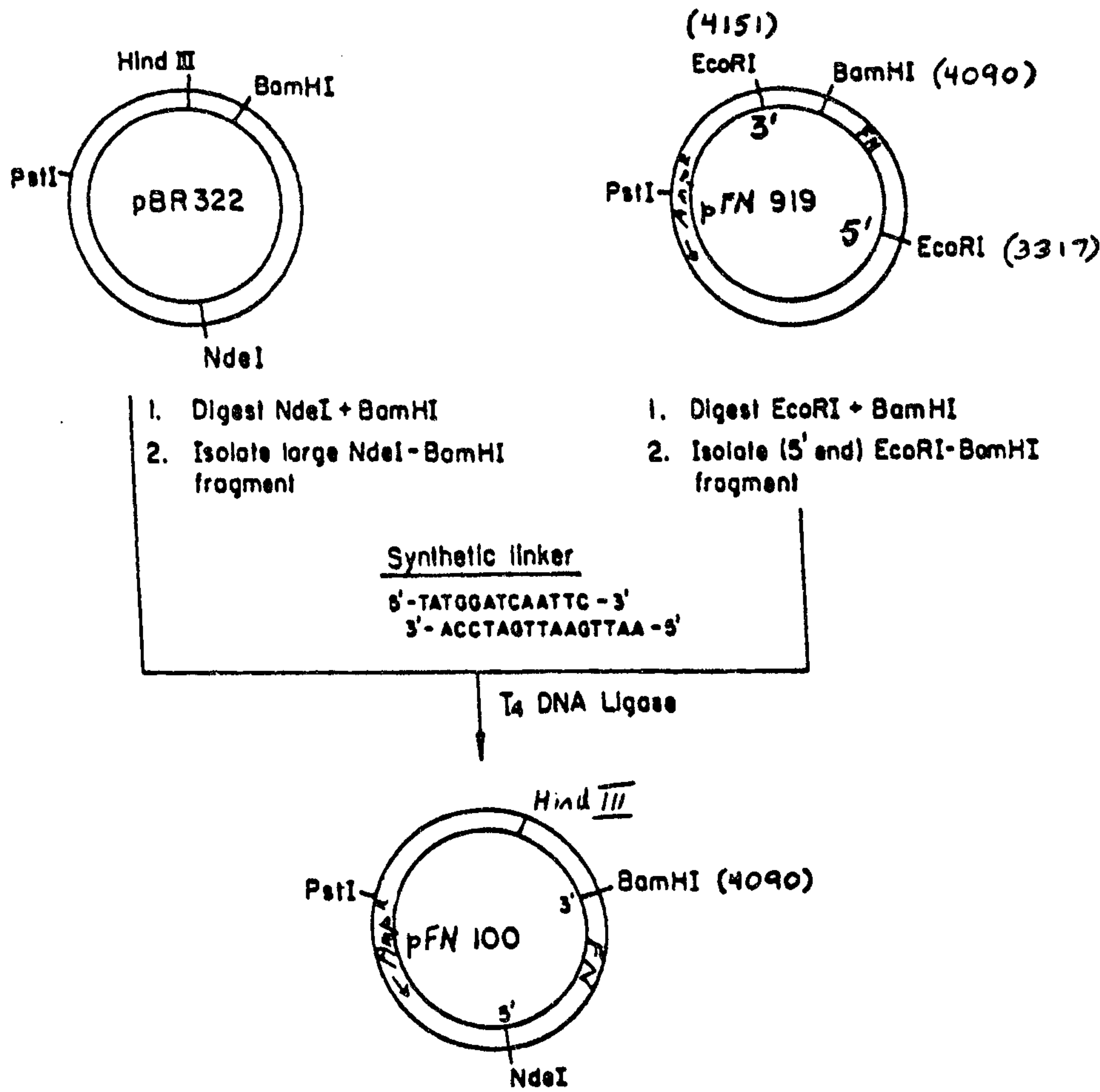
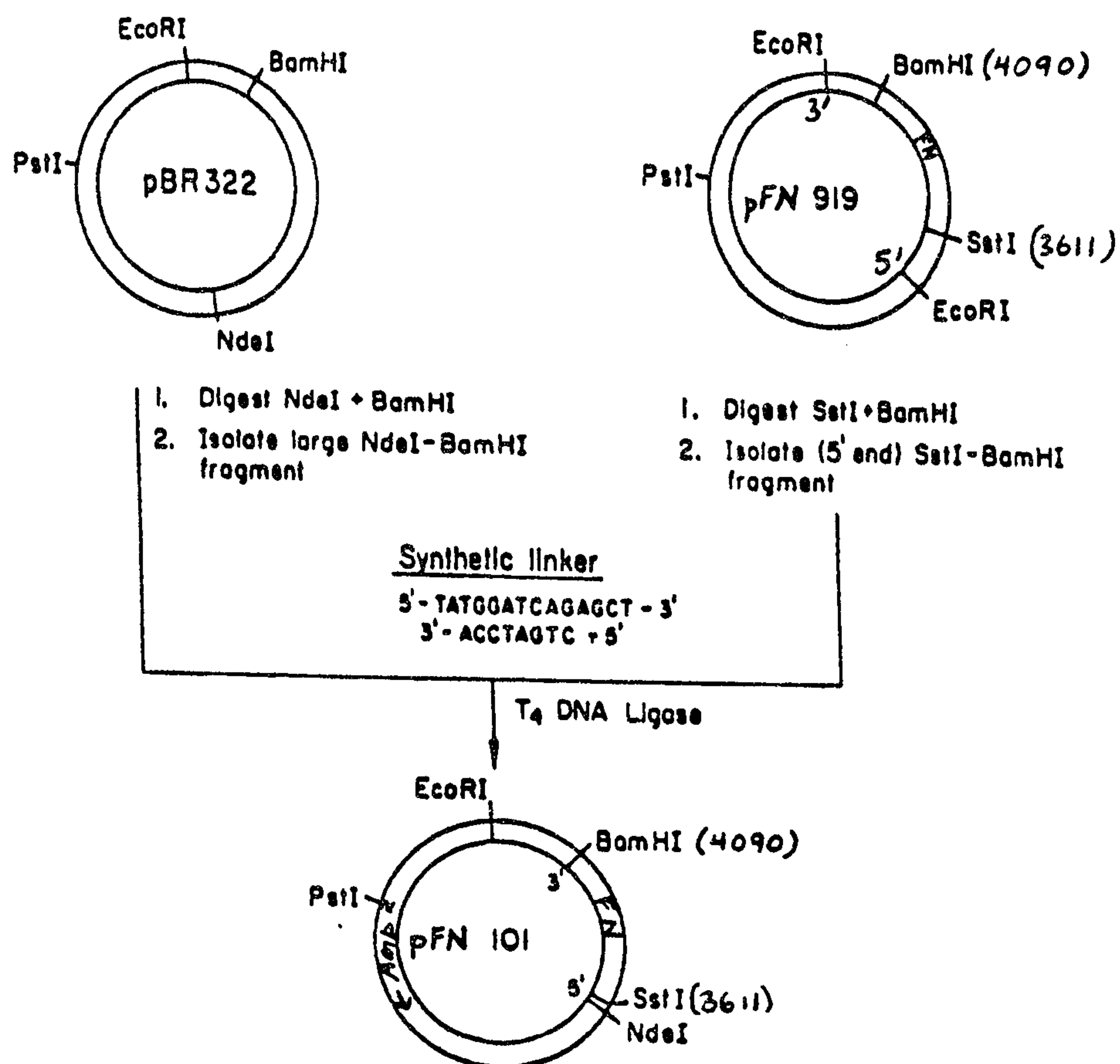


Figure 2



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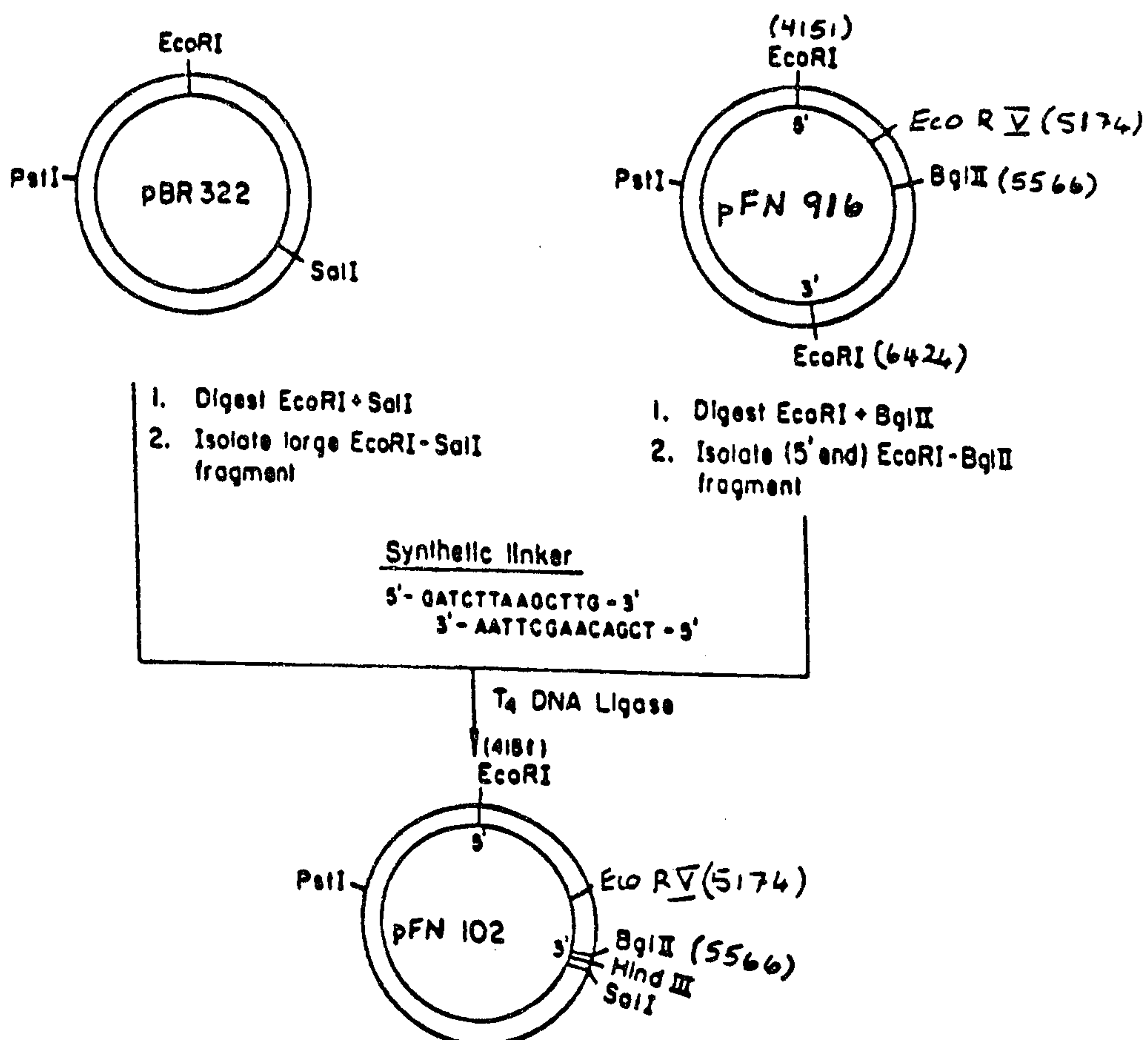
Figure 3



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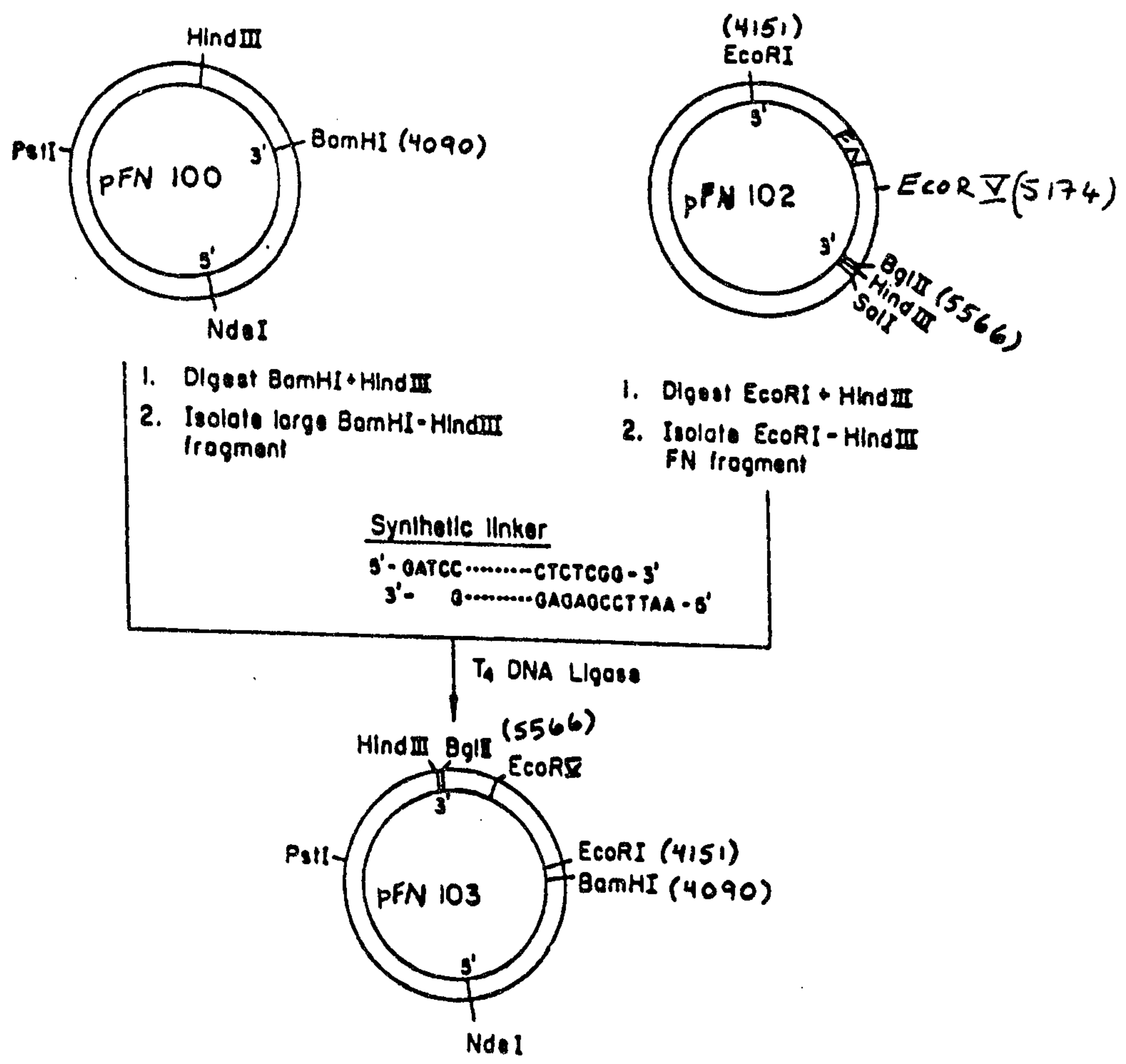
Figure 4



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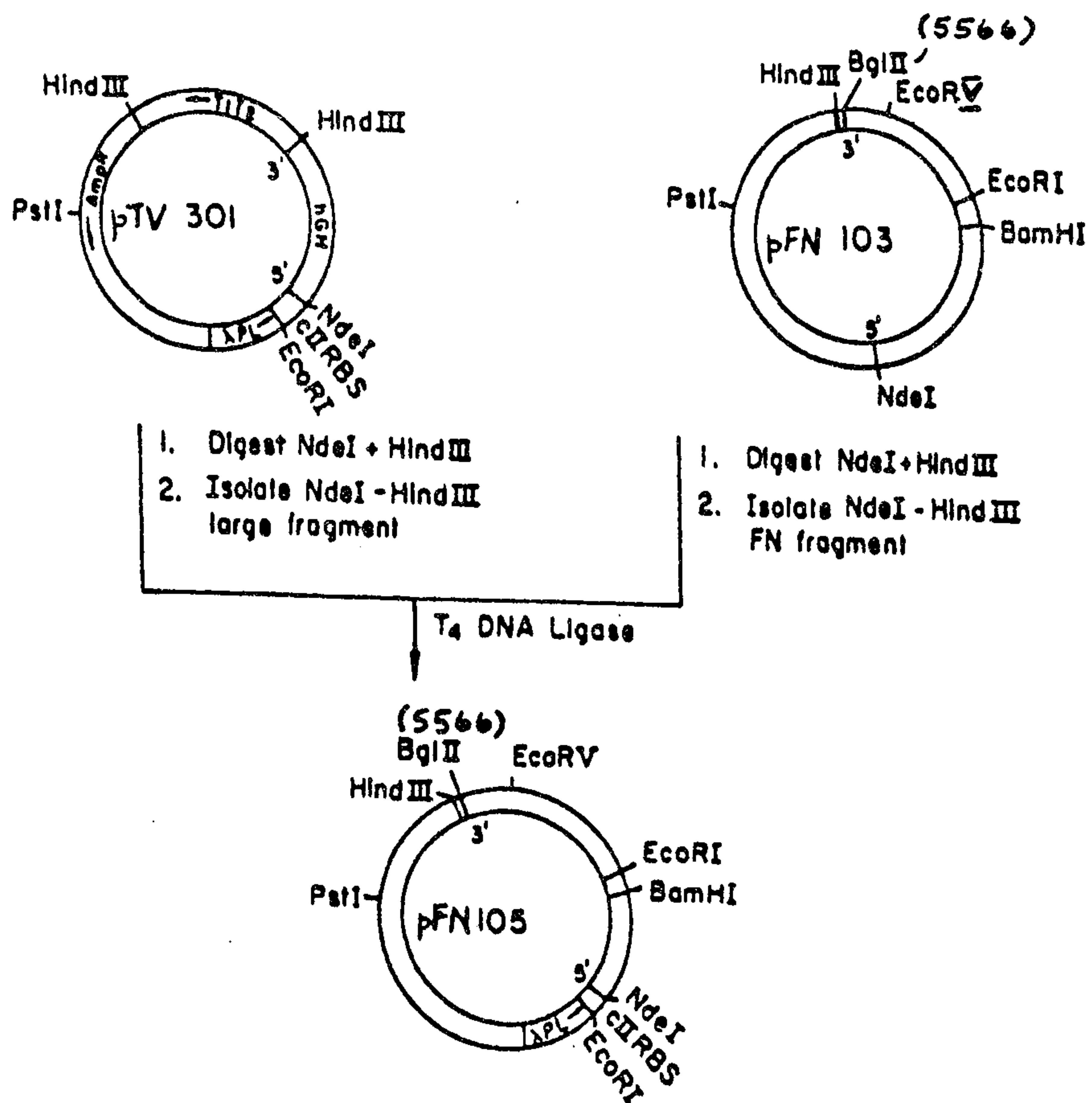
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Figure 5



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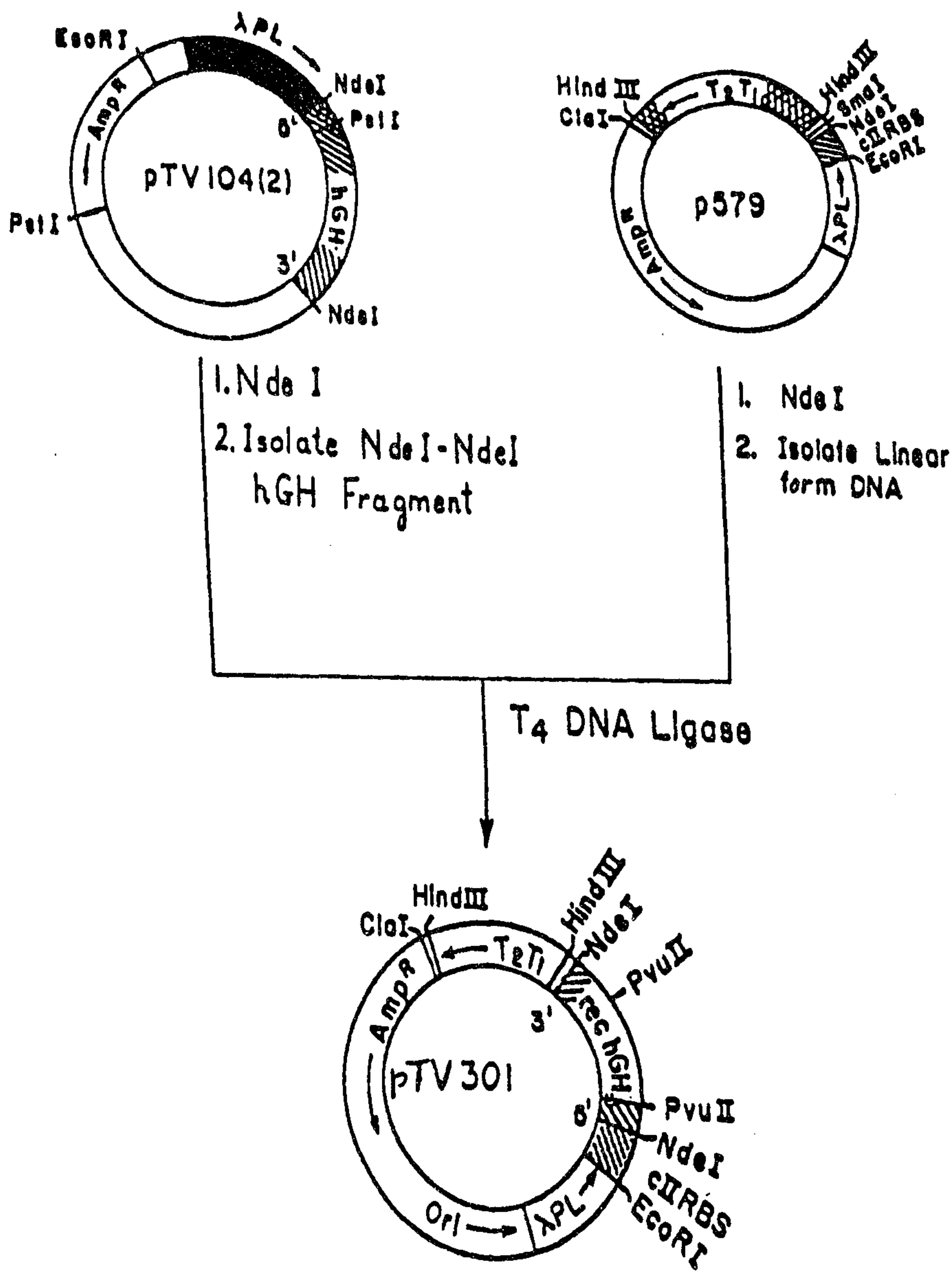
Figure 6



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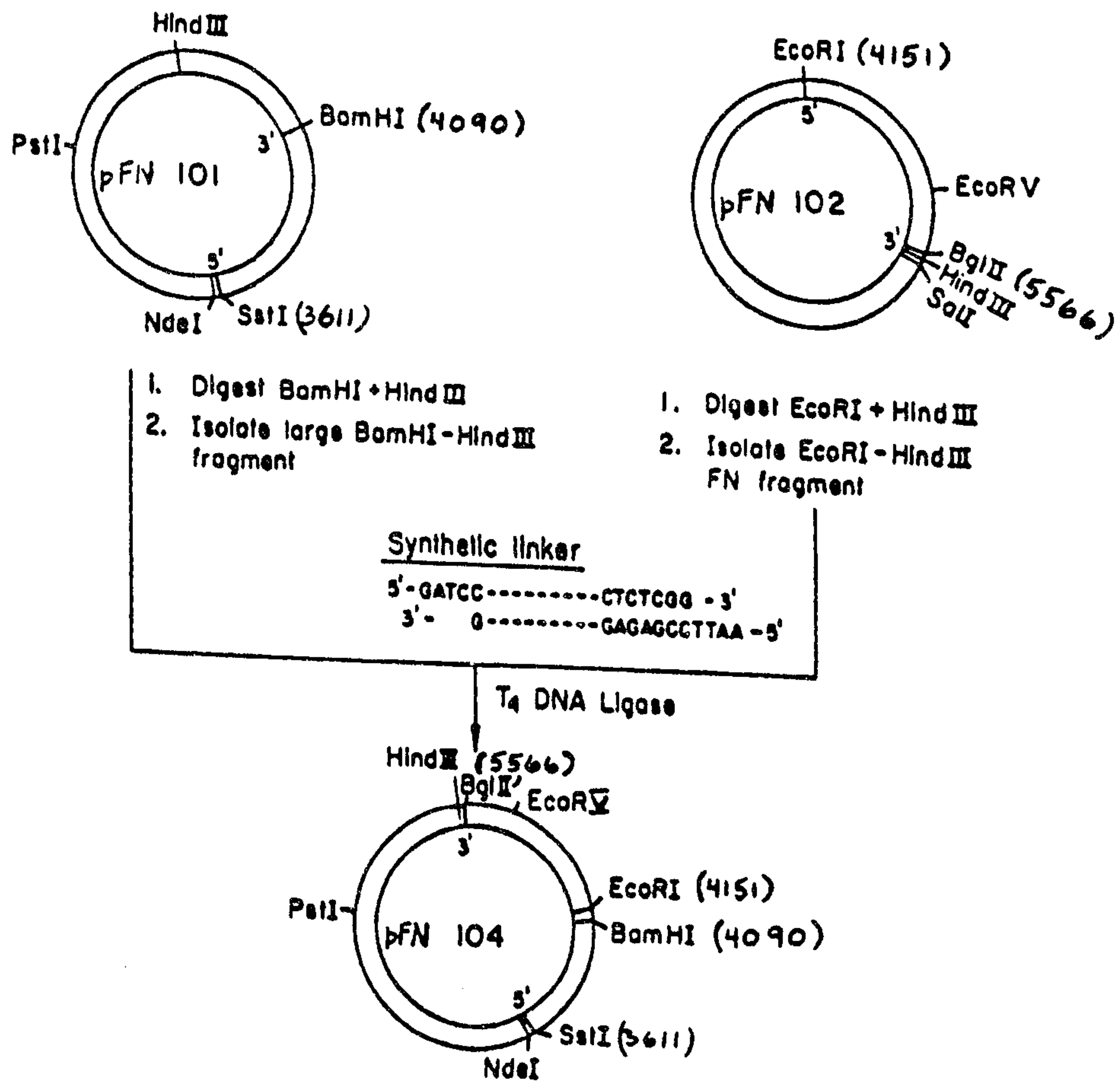
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Figure 7



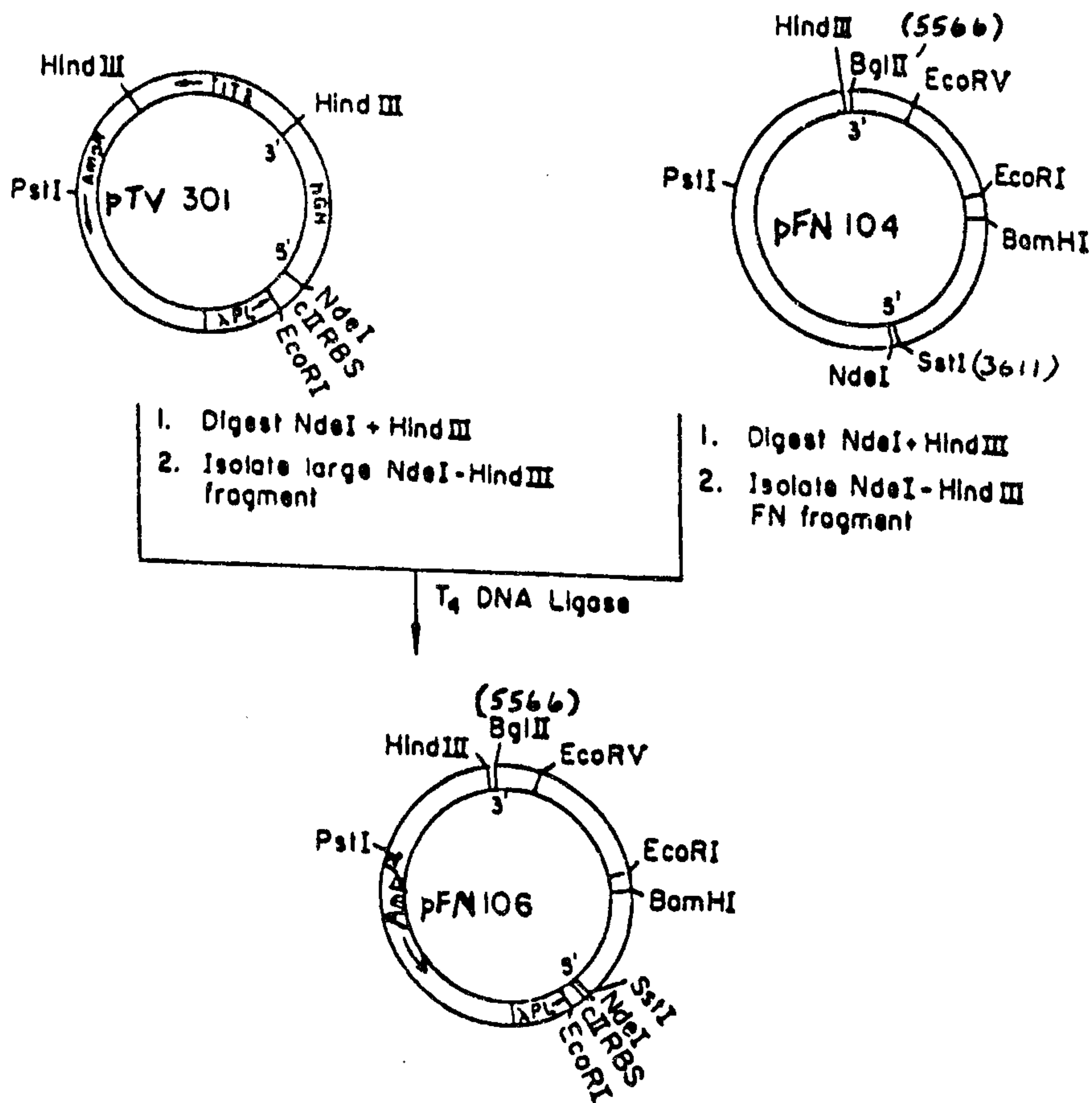
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Figure 8

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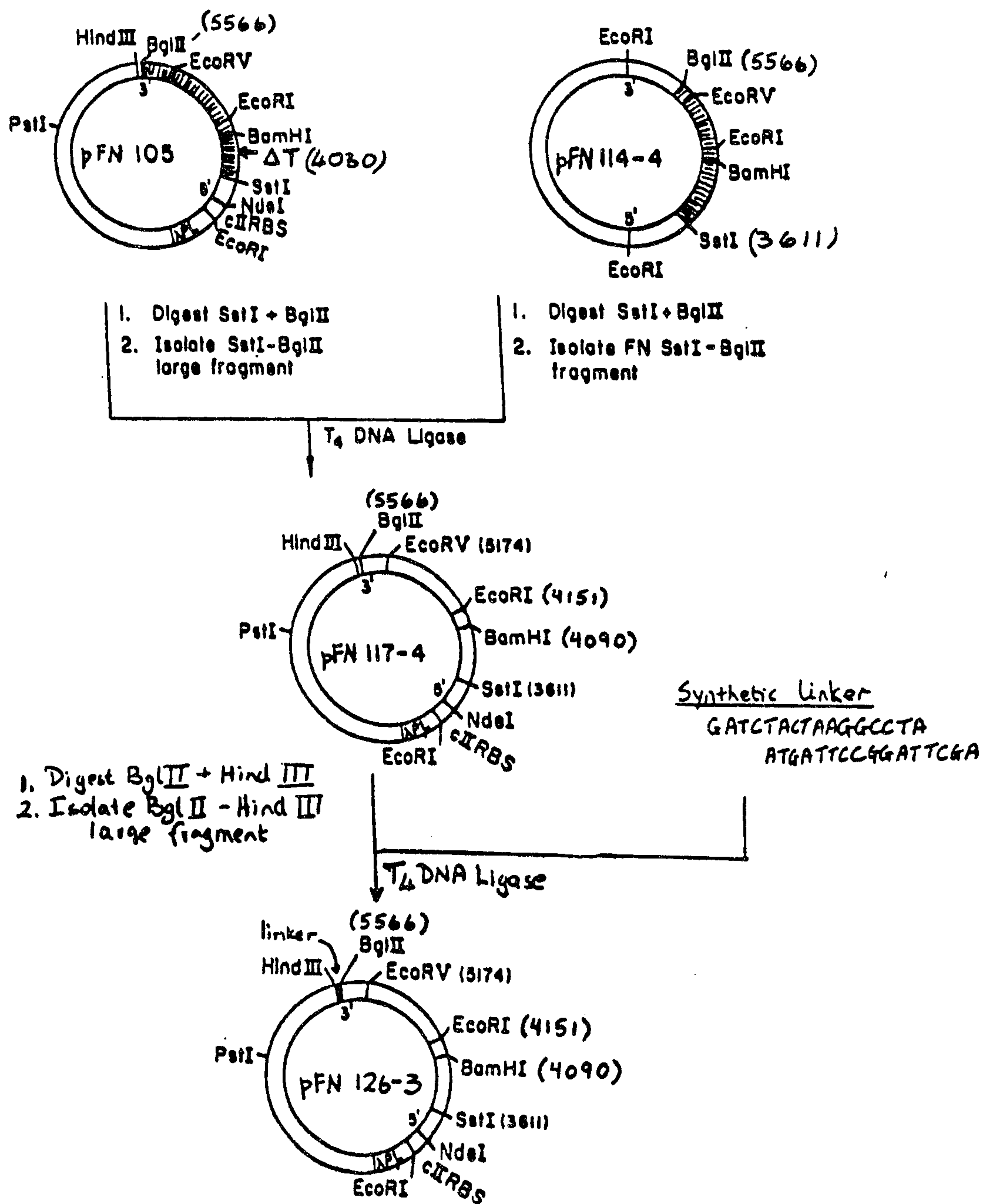
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Figure 9



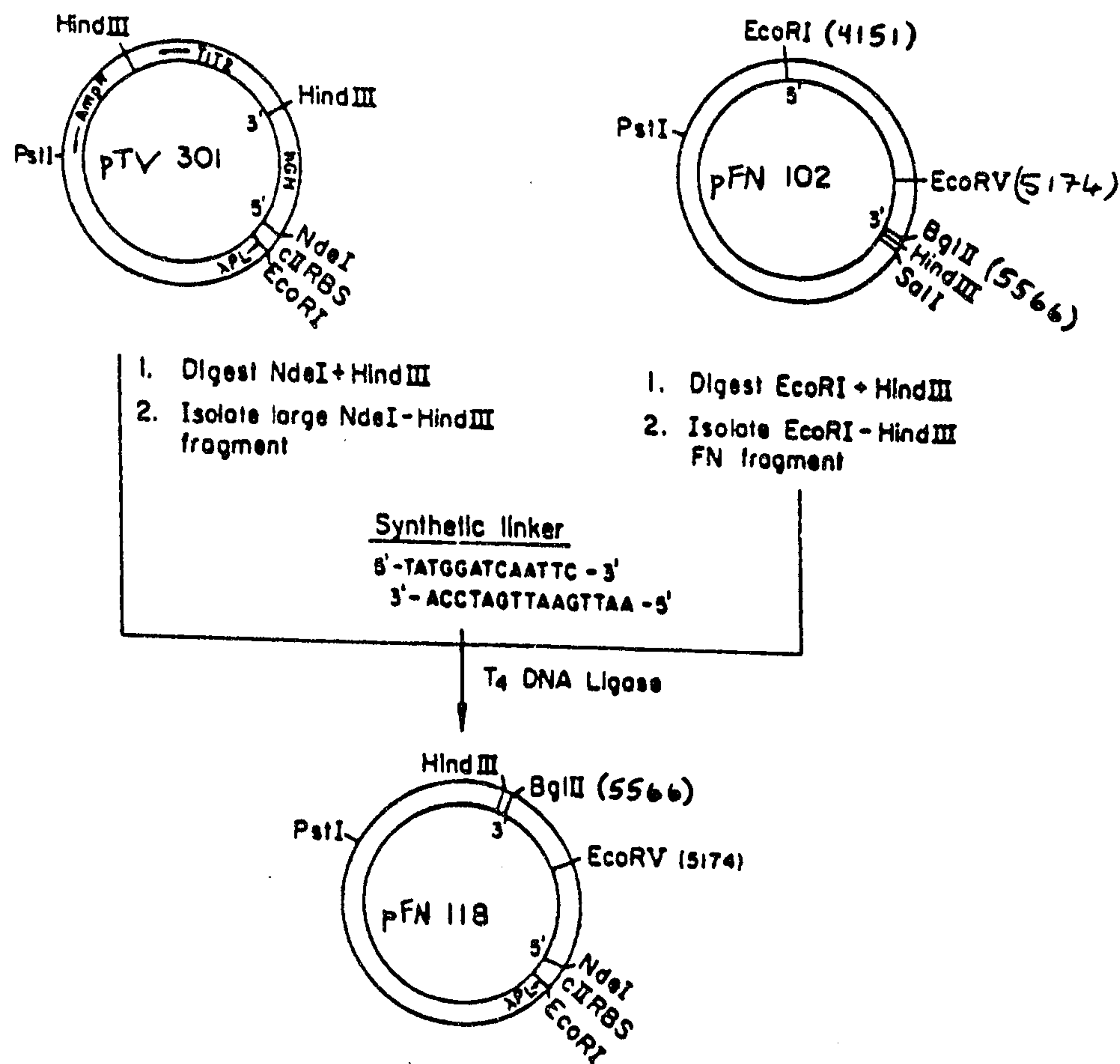
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Figure 10



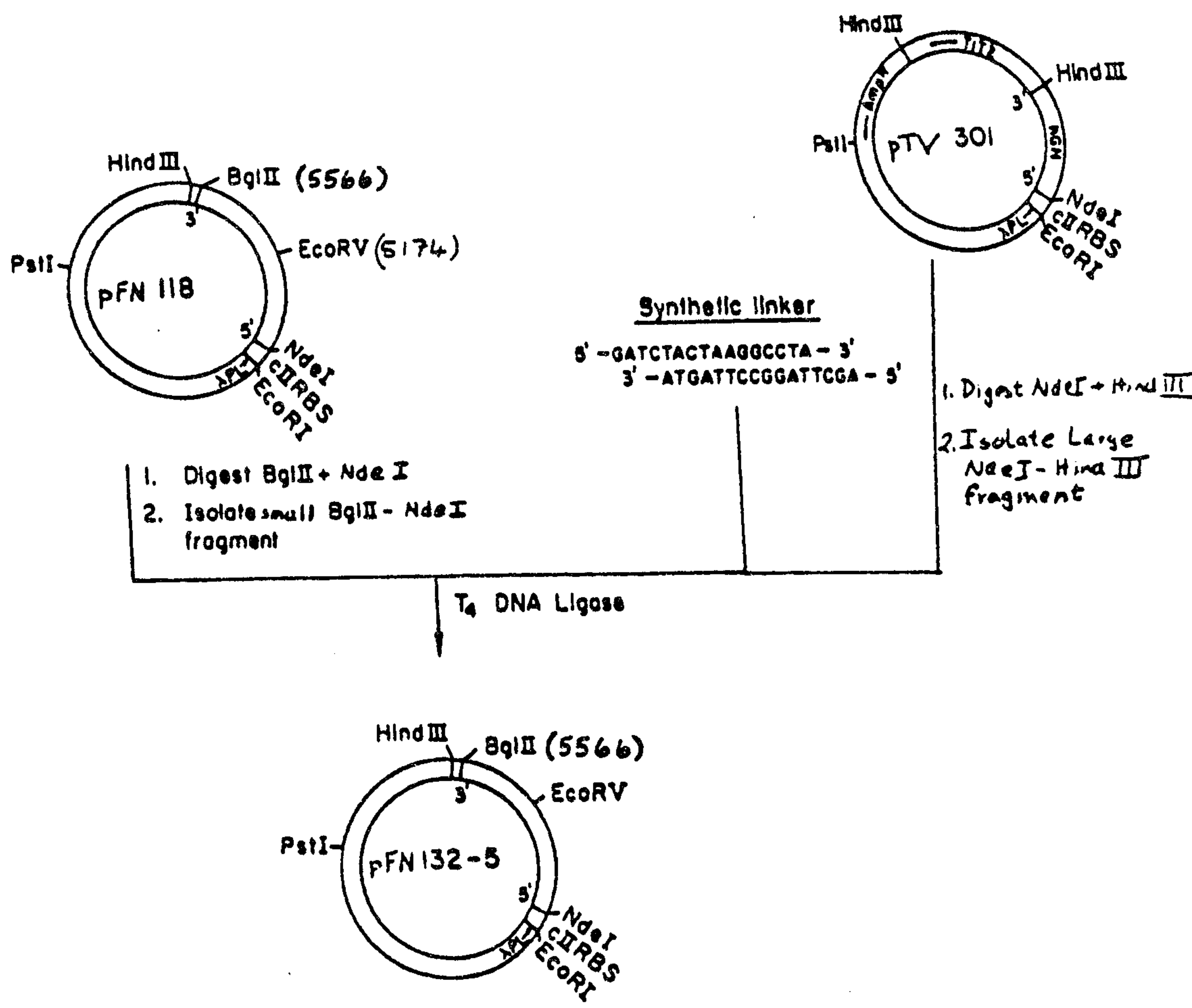
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Figure 11



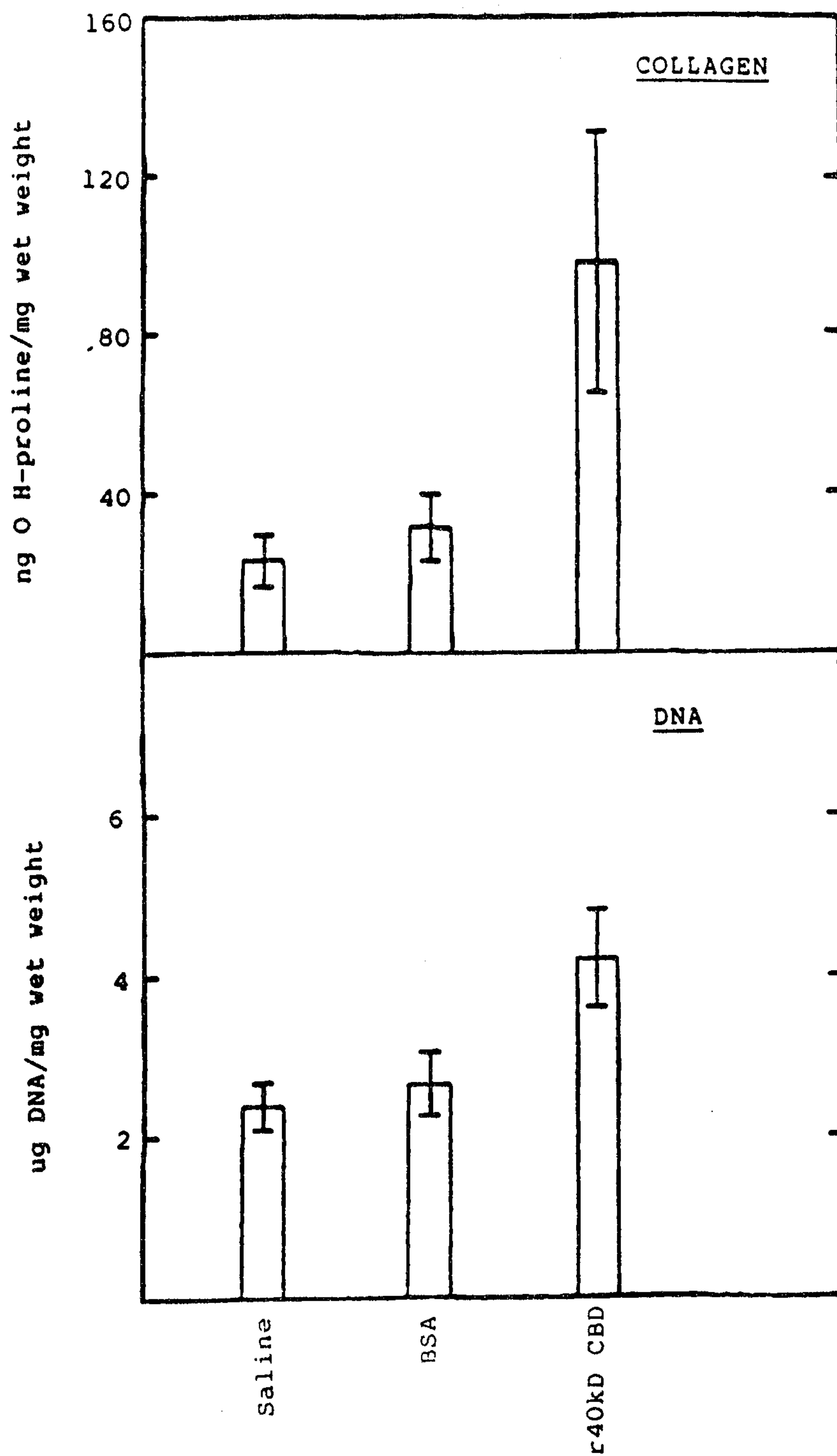
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Figure 12



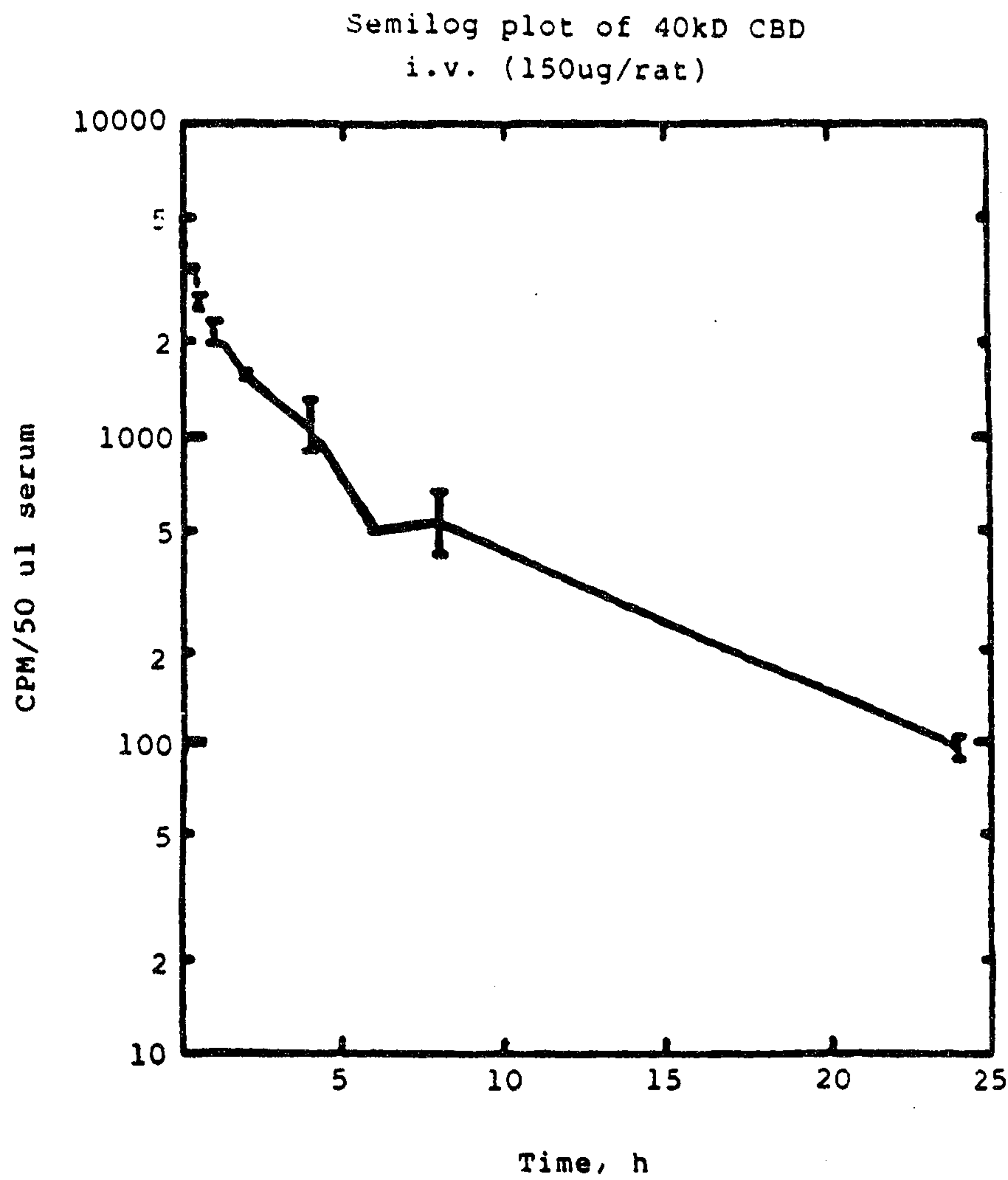
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Figure 13

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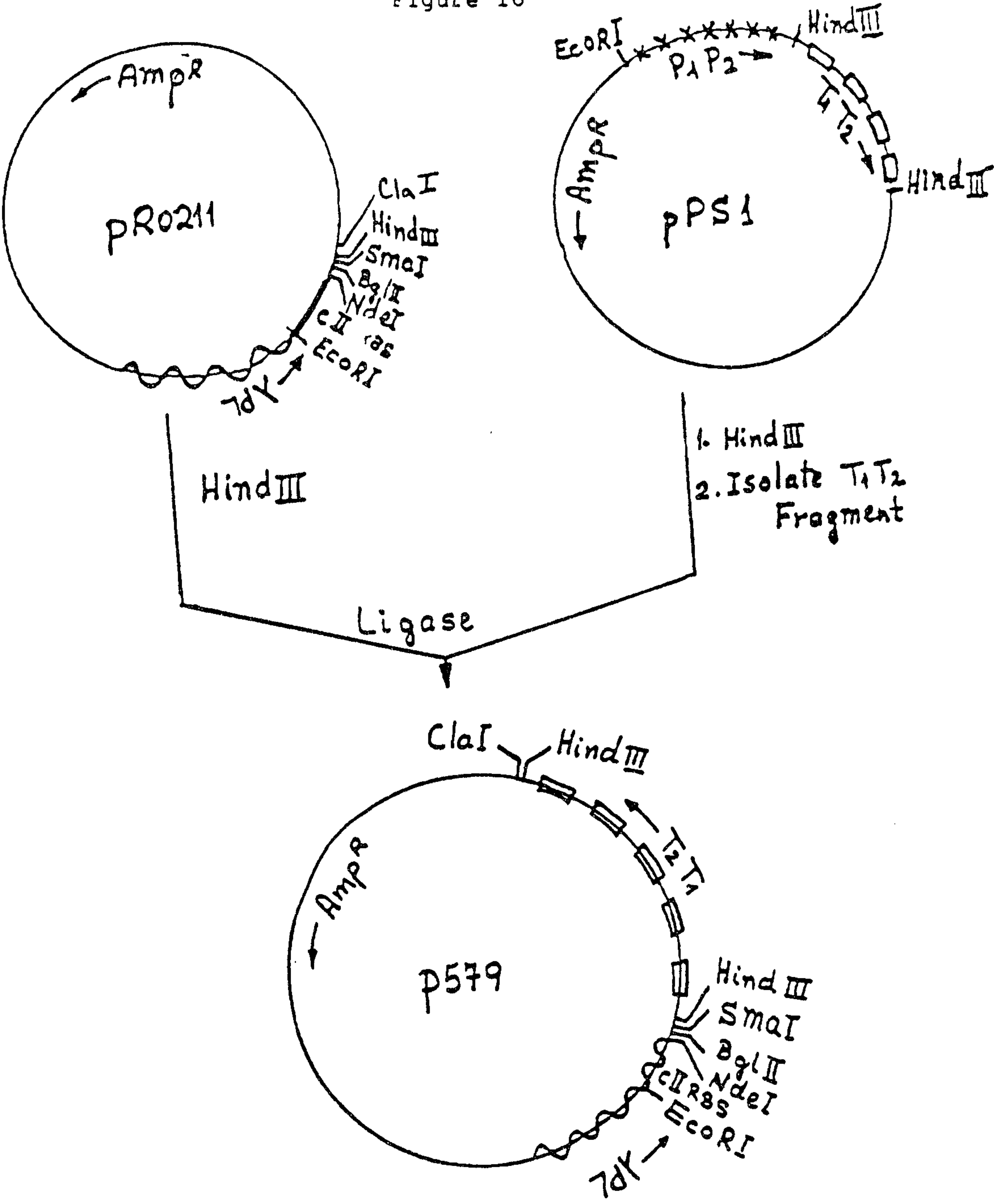
Figure 14



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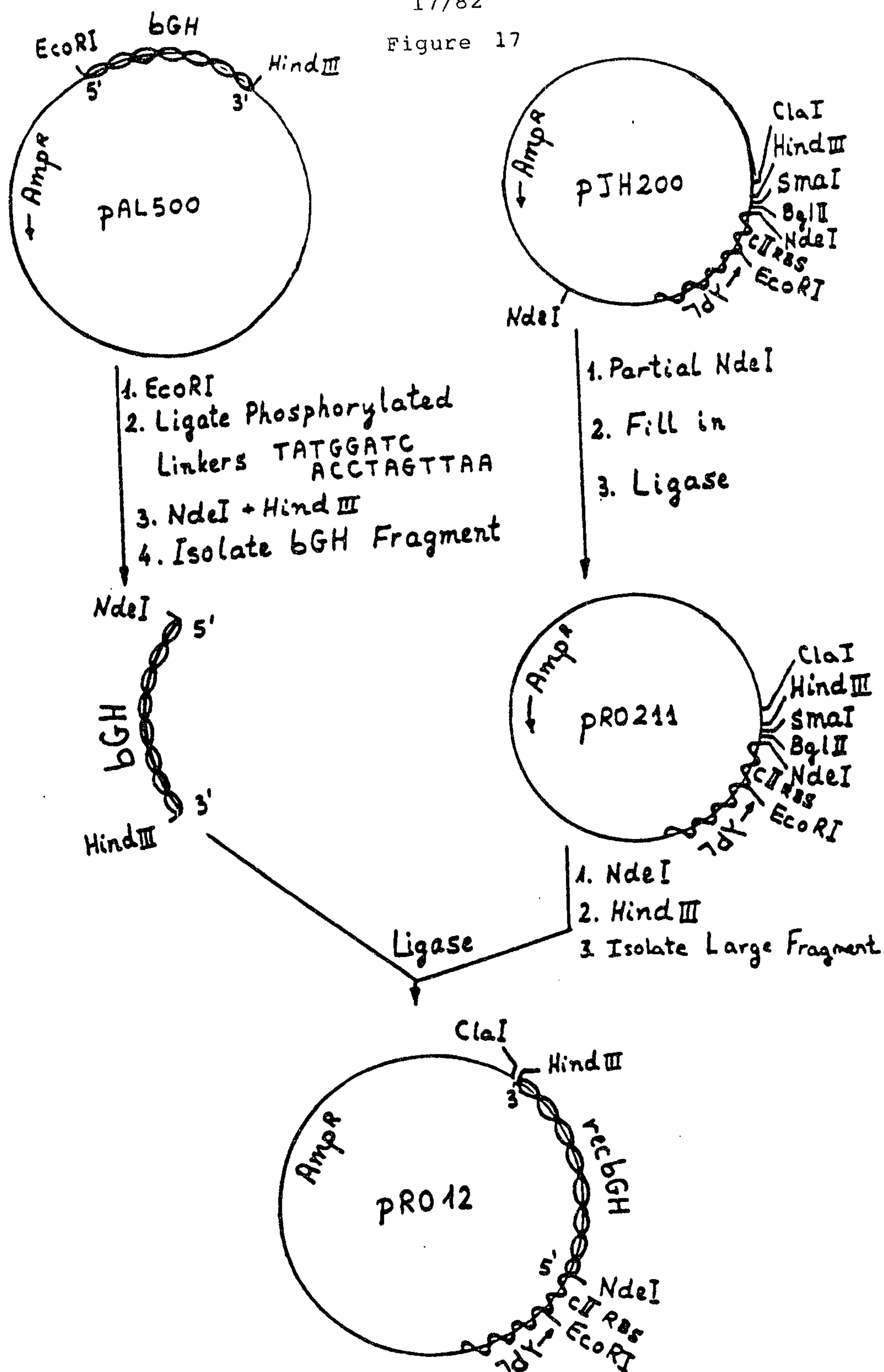
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Figure 16



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Figure 17

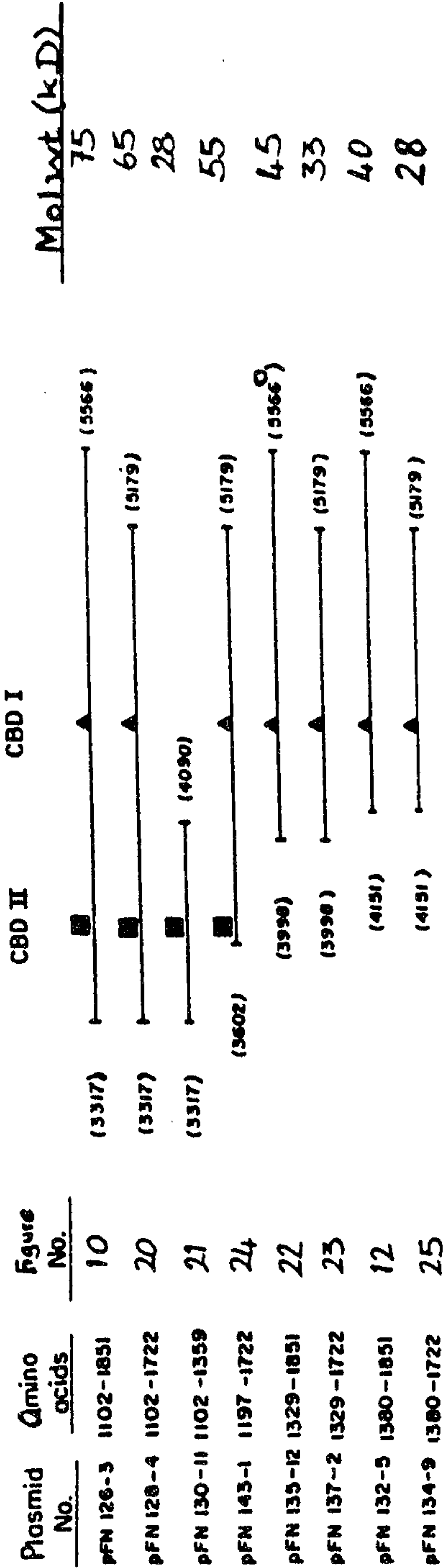


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Figure 18

Recombinant - Cell Binding Domain (r-CBD)



o denotes C - terminus with additional non - fibronectin derived amino acids

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Figure 19

PFN RECOMBINANT FRAGMENTS

plasmid name (PFN)	nucleotide ¹ "kD" from-to	nu. ² no.	a.a. ³ no.	a.a. sequence termini	a.a. ⁴ from-to	MWt (kD) calcd.
126-3	75 3317-5566	1980	660	^a SDSGS....DYKIY	1102-1851	72.6
128-4	65 3317-5179	1593	531	^a SDSGS....LTGYR	1102-1722	58.4
130-11	28 3317-4090	774	258	^a SDSGS....TCYRI	1102-1359	28.0
143-1	55 3602-5179	1308	436	^b DQSSC ...LTGYR	1197-1722	48.0
135-12	45 3998-5566	1299	433	^b SPTGI....DYKIY	1329-1851	47.6
137-2	33 3998-5179	912	304	^b SPTGI....LTGYR	1329-1722	33.4
132-5	40 4151-5566	1146	382	^c NSITL....DYKIY	1380-1851	42.0
134-9	28(M) 4151-5179	759	253	^c NSITL....LTGYR	1380-1722	27.8
authentic	75 2630-4678	2049	683	SDTVP....PGPTK	873-1555	75.1

- ^a MDQFNS +....
- ^b (M) + (probably cleaved)
- ^c MDQF +....

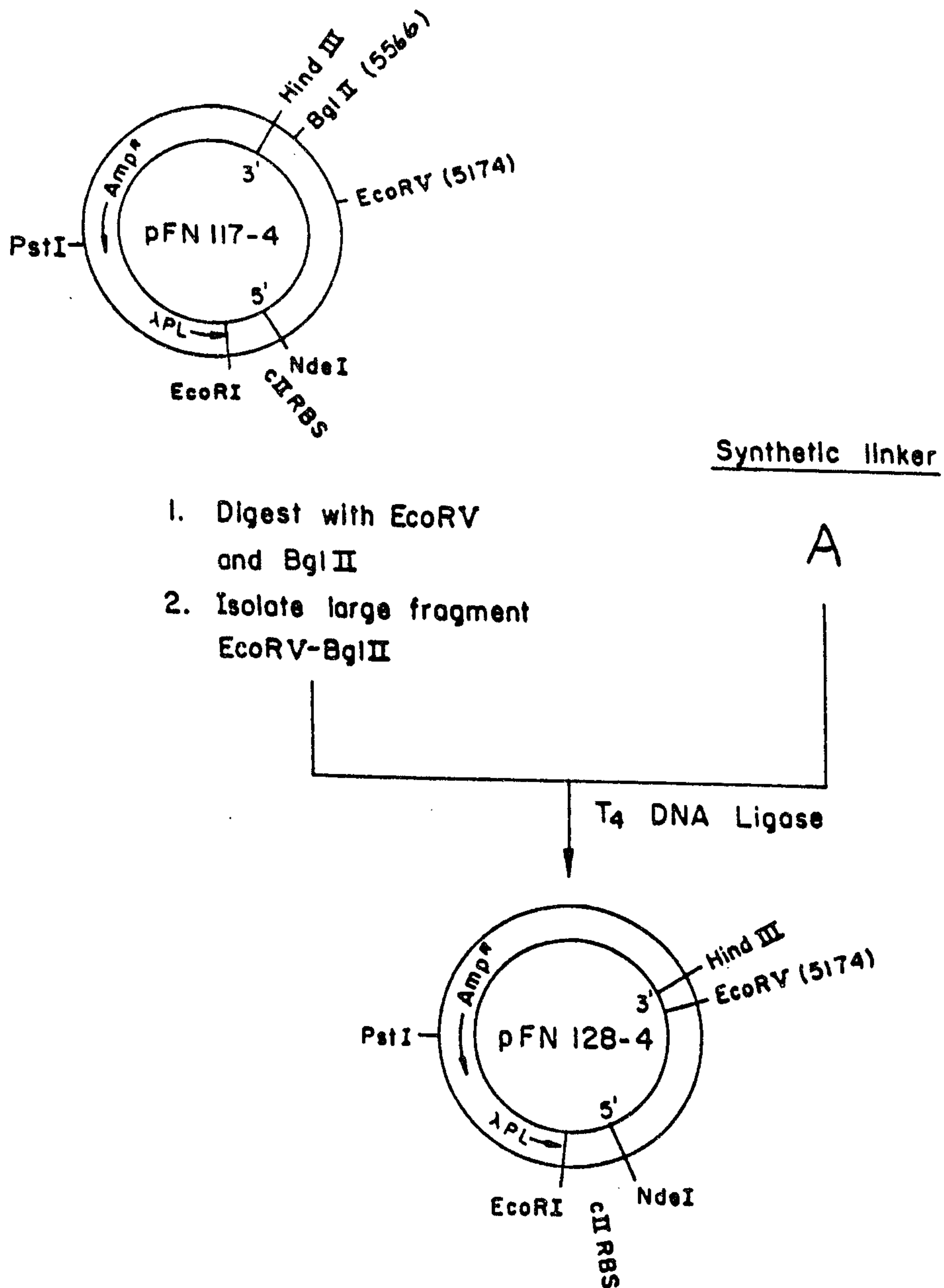
¹ Nucleotide sequence from Barralle's Patent EPO no. 207751.
² 270 Nucleotides (nu), corresponding to the extra domain (ED) are missing. These are nos. 4812-5081, i.e. starting with ACATT and ending with TCCACAG.
³ 90 Amino acids (a.a.), corresponding to the ED, are missing. These are nos. 1600-1689, i.e. starting with NIDRQ and ending with GTQST.
⁴ Amino acid sequence starts at nucleotide no. 14.

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Figure 20

Construction of clone pFN 128-4 (a.a. 1102→1722)

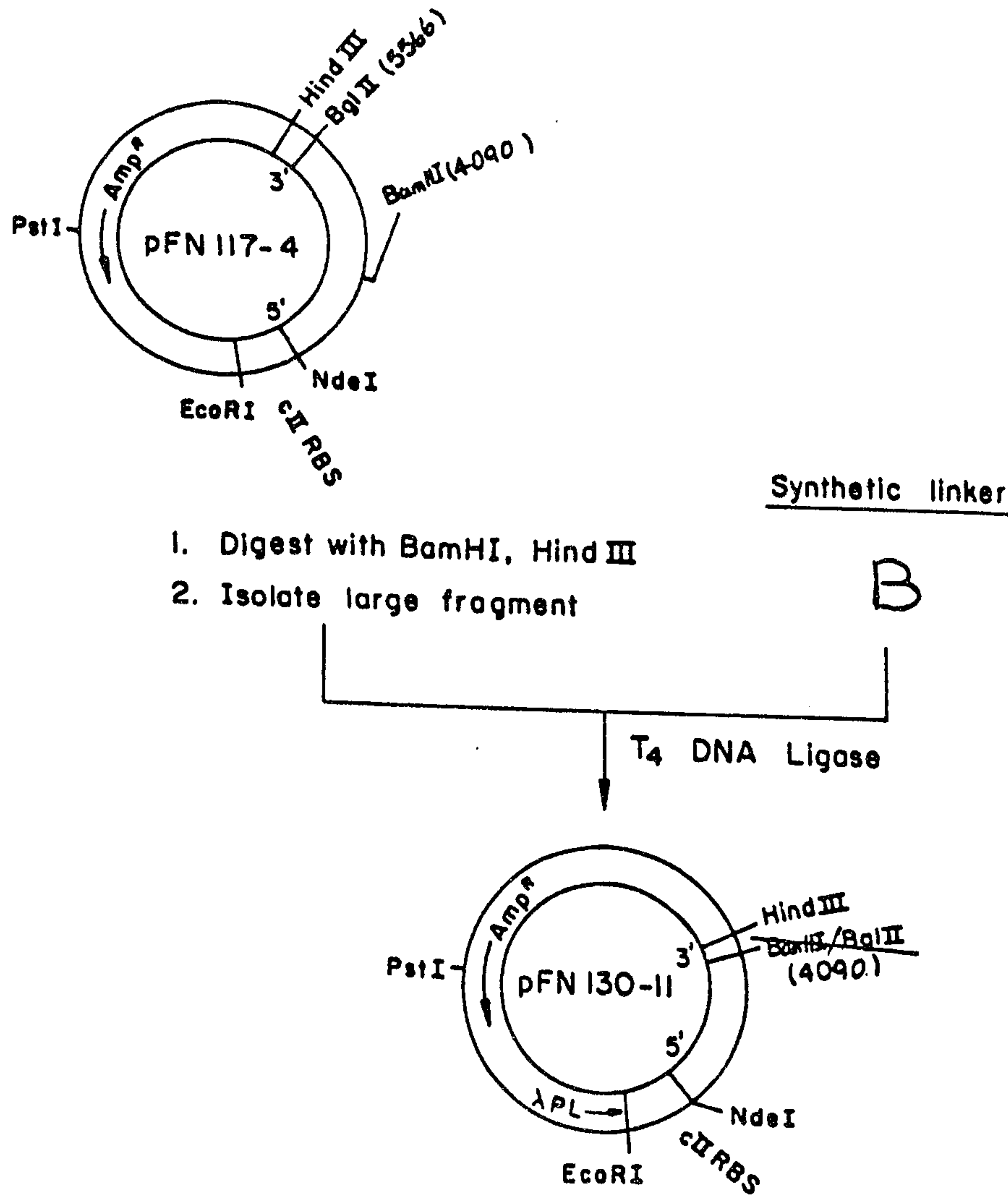


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Figure 21

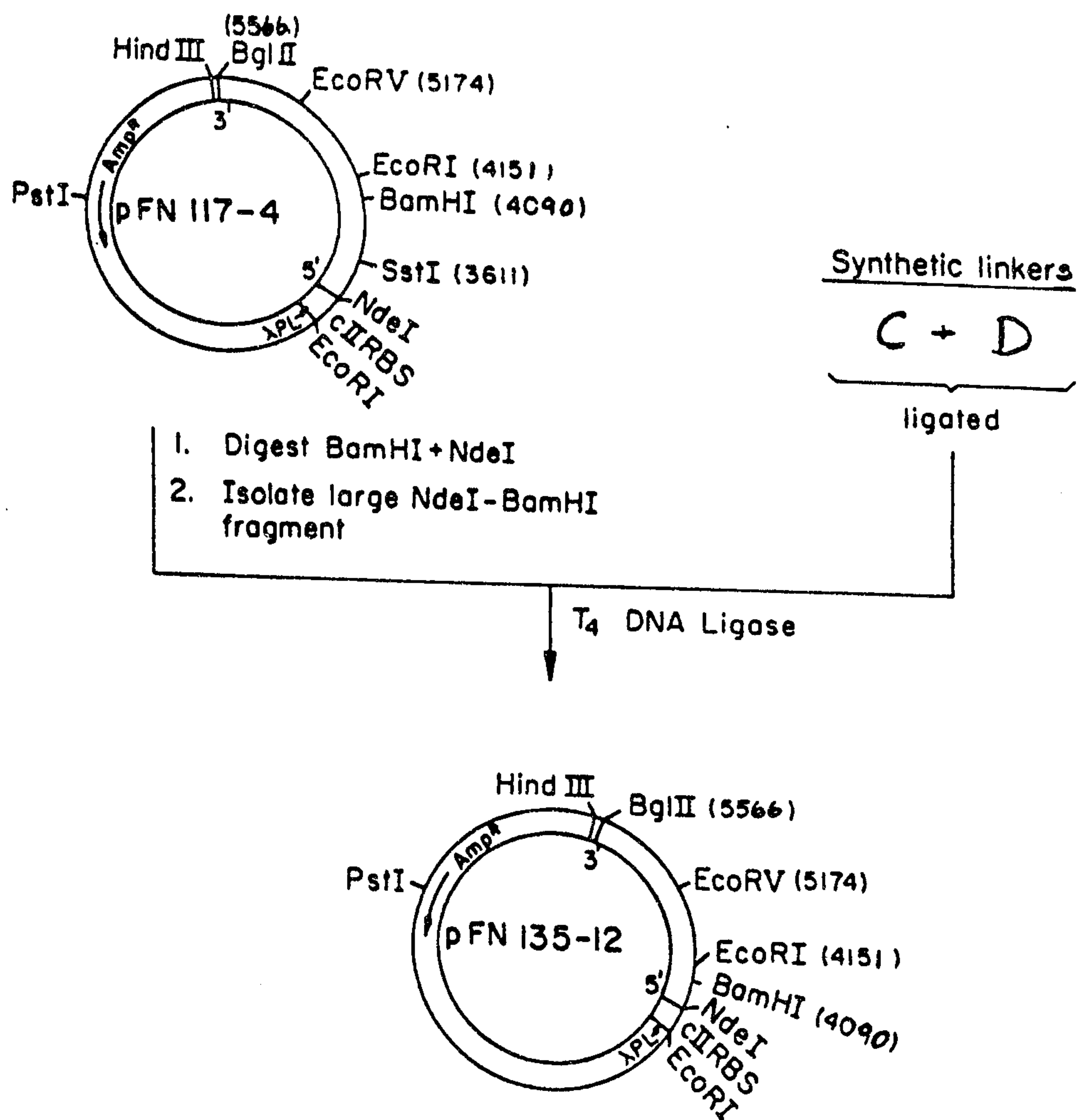
Construction of clone pFN 130-11
(a.a. 1102 → 1359)



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Figure 22

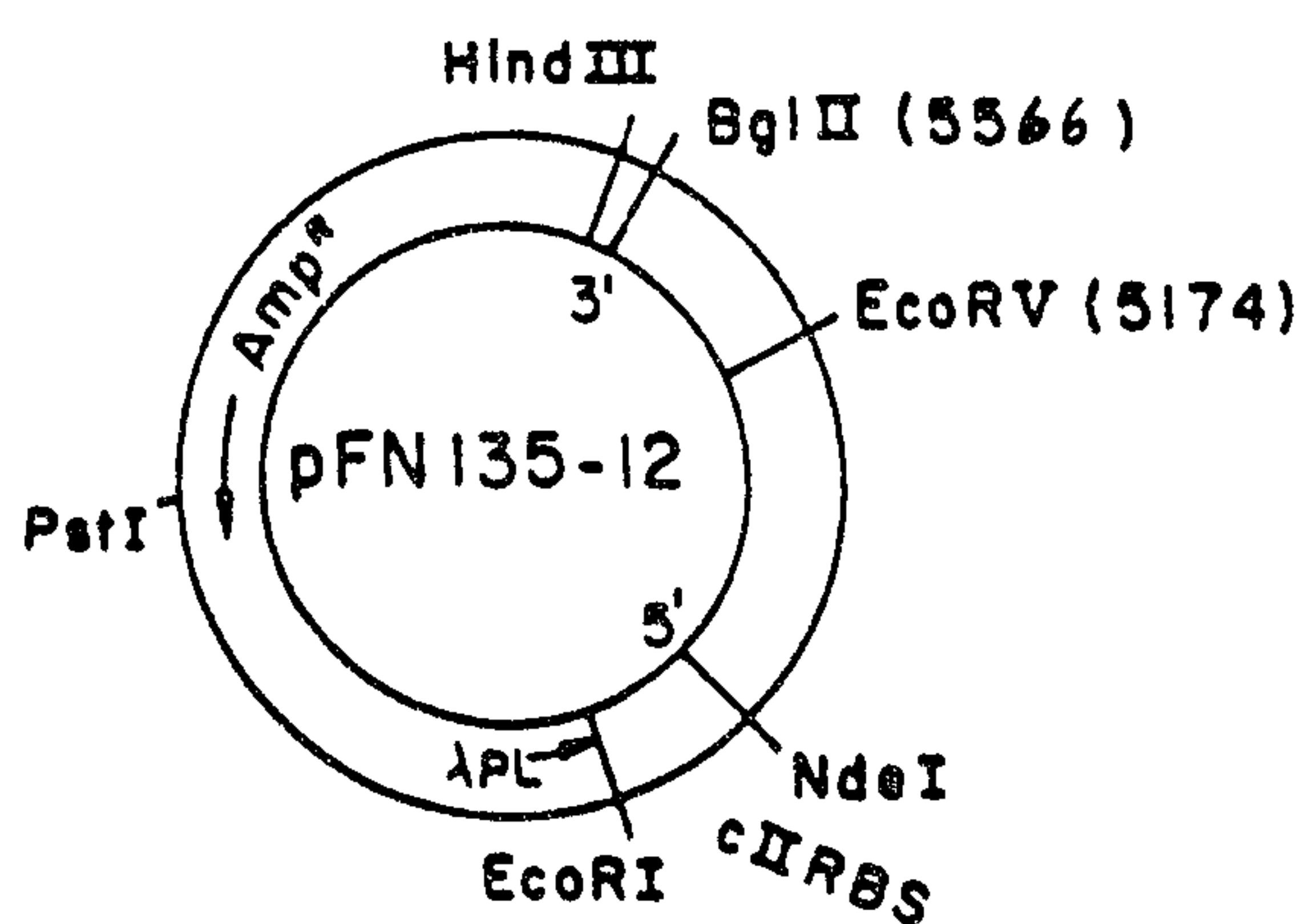


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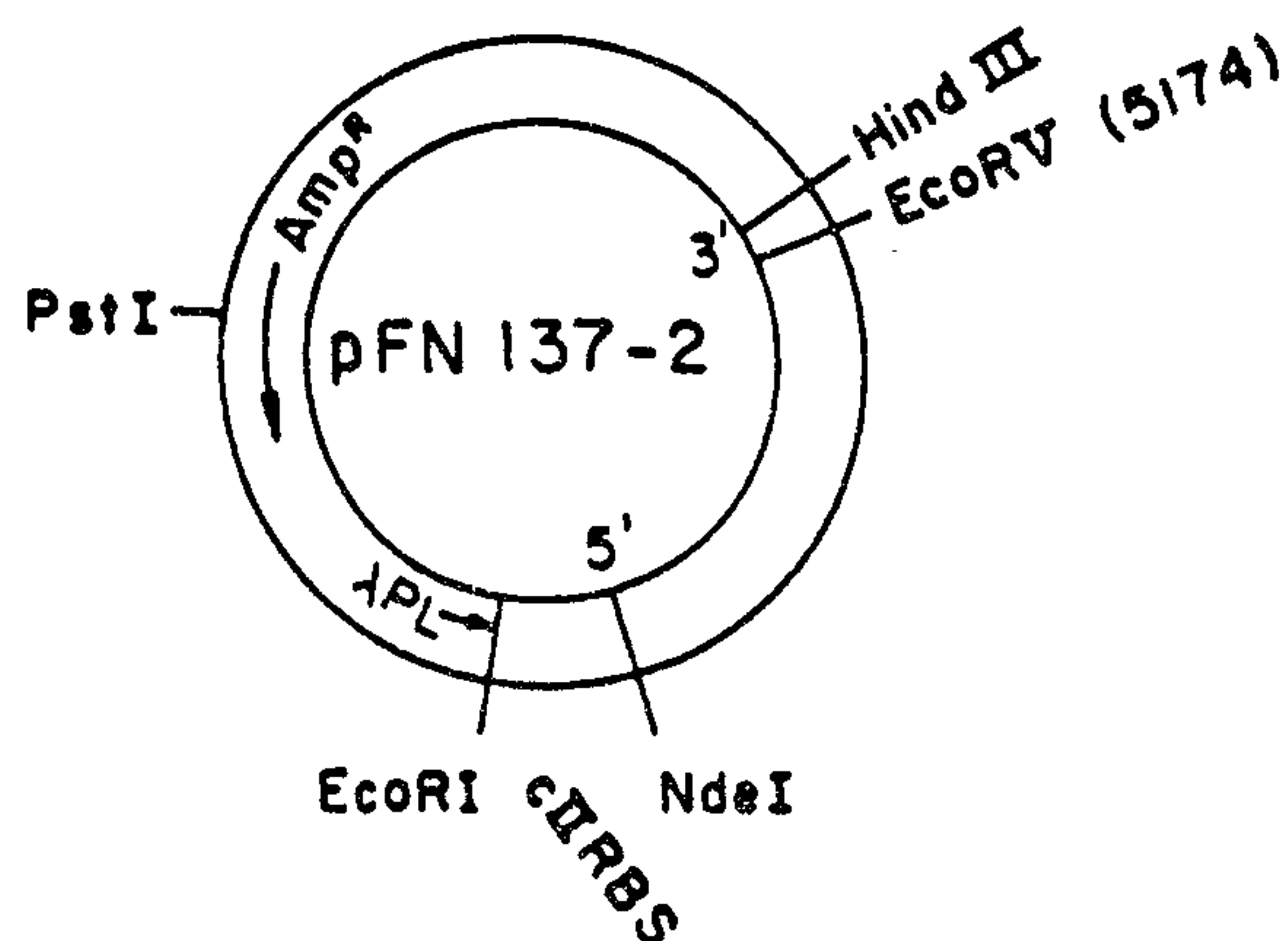
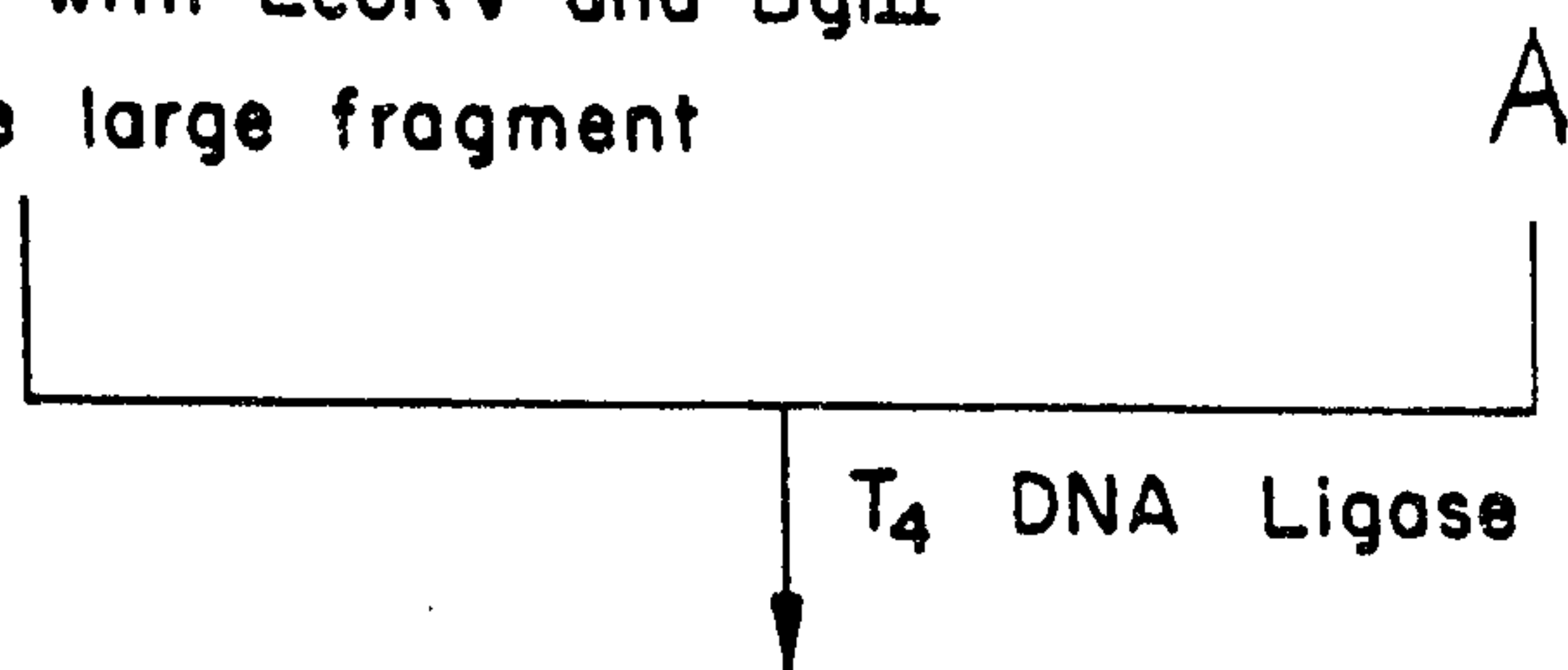
Figure 23

Construction of clone pFN 137-2
(a.a. 1329→1722)



Synthetic linker

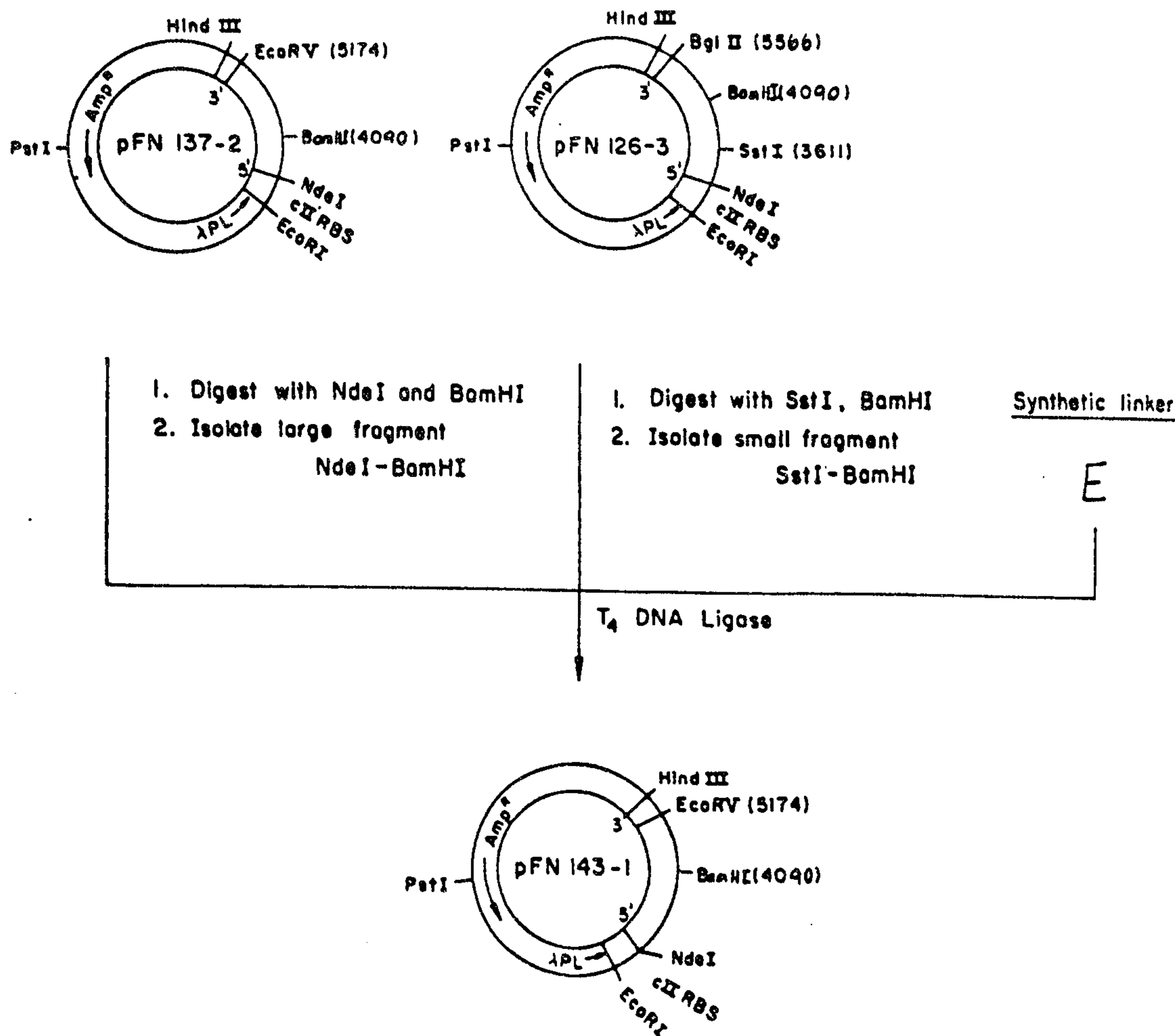
1. Digest with EcoRV and Bgl II
2. Isolate large fragment



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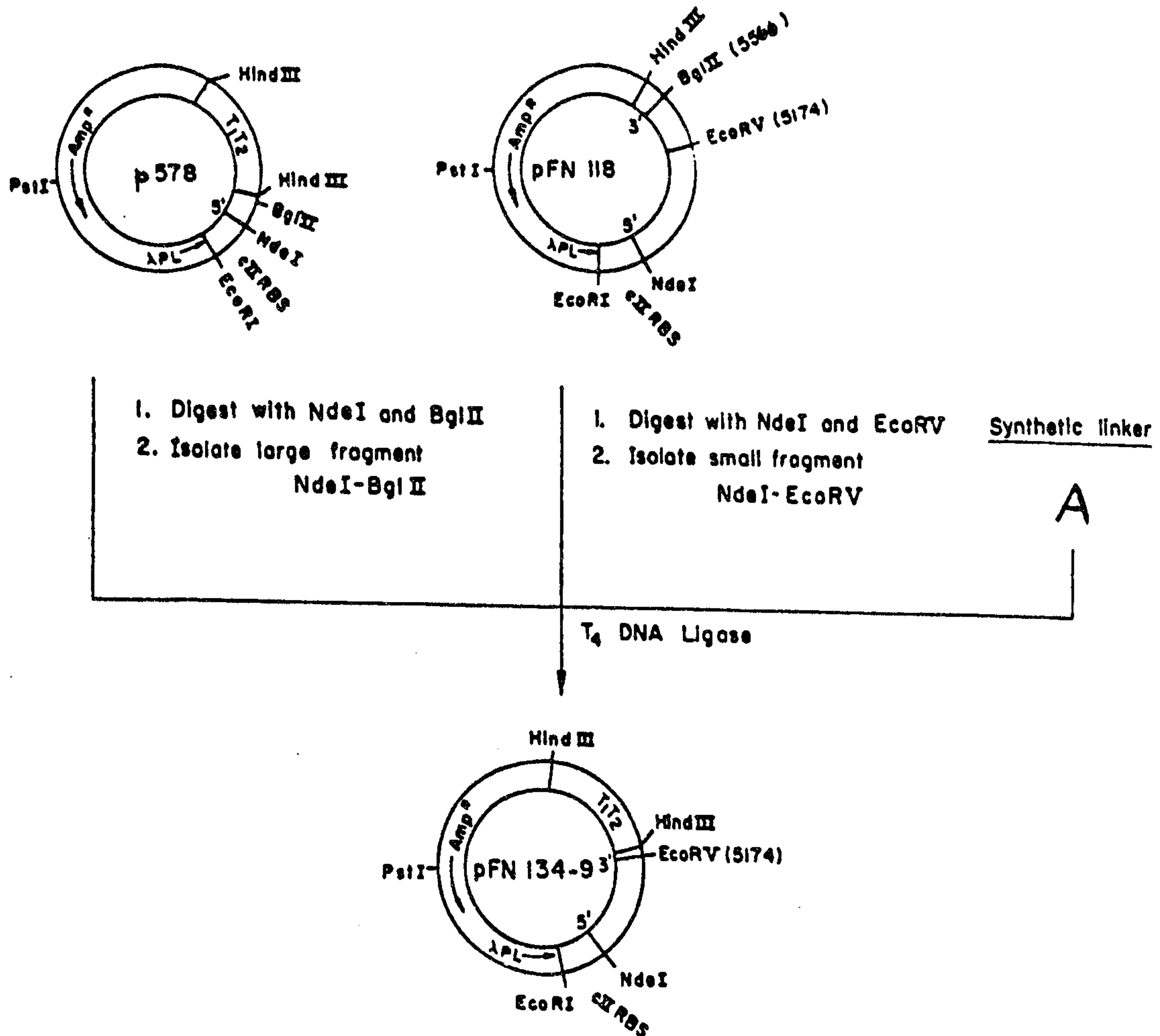
Figure 24



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Figure 25

Construction of clone pFN 134-9
(a.a. 1380-1722)



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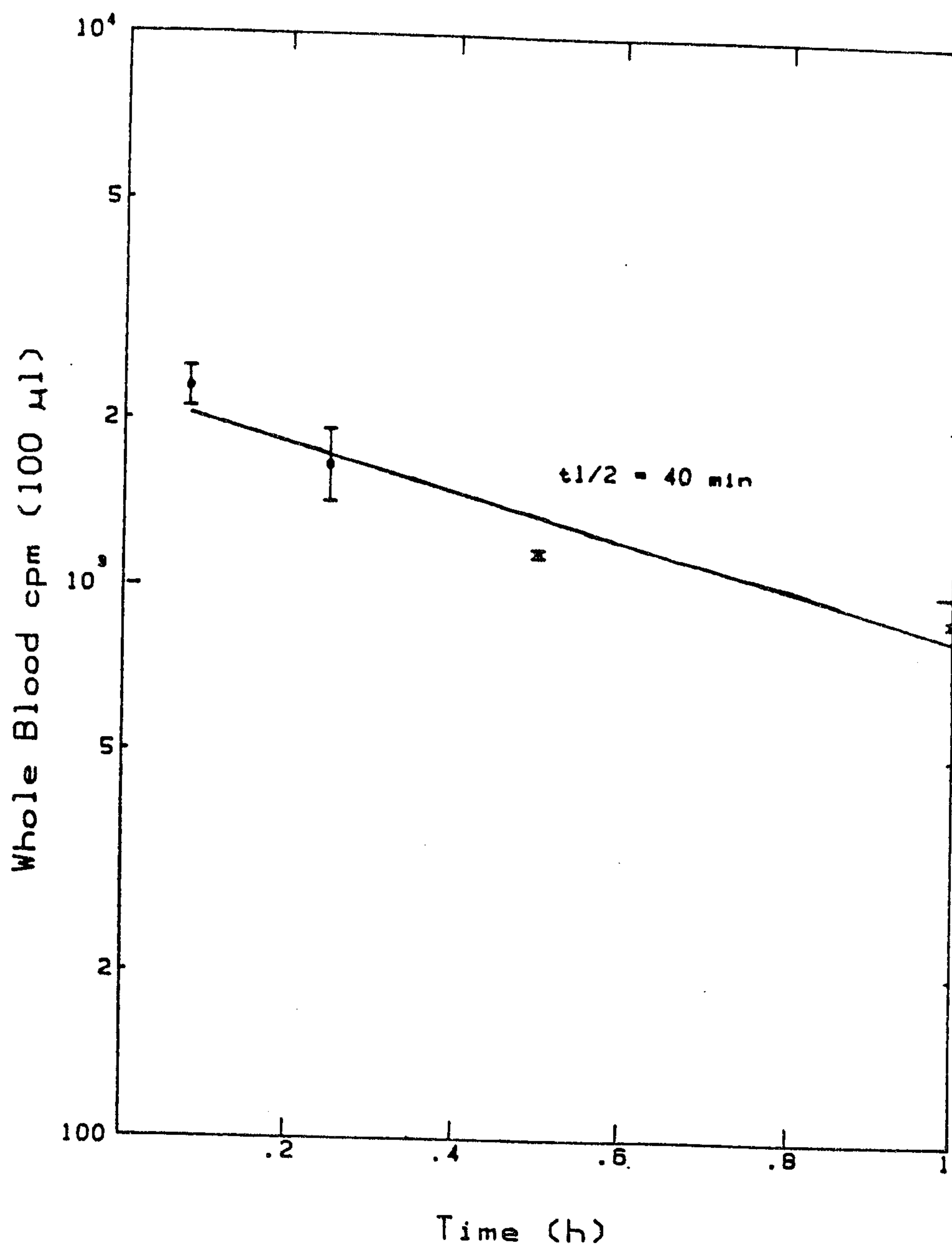
Figure 26Synthetic Linkers Used in the Construction of Plasmids

- A. 5' - ATCGATAAGCTTA - 3'
3' - TAGCTATTCGAATCTAG - 5'
- B. 5' - GATCTACTAAGGCCTA - 3'
3' - ATGATTCCGGATTCTGA - 5'
- C. 5' - TATGTCCCCAACTGGCATTGACTTTTCTGATATTACTGCCAACTCTTTTACTGTG - 3'
3' - ACAGGGGTTGACCGTAACTGAAAAGACTATAATGACGGTTGAGAAAAT - 5'
- D. 5' - CACTGGATTGCTCCTCGAGCCACCATCACTGGCTACAG - 3'
3' - GACACGTGACCTAACGAGGAGCTCGGTGGTAGTGACCGATGTCCTAG - 5'
- E. 5' - TATGGATCAGAGCT - 3'
3' - ACCTAGTC - 5'

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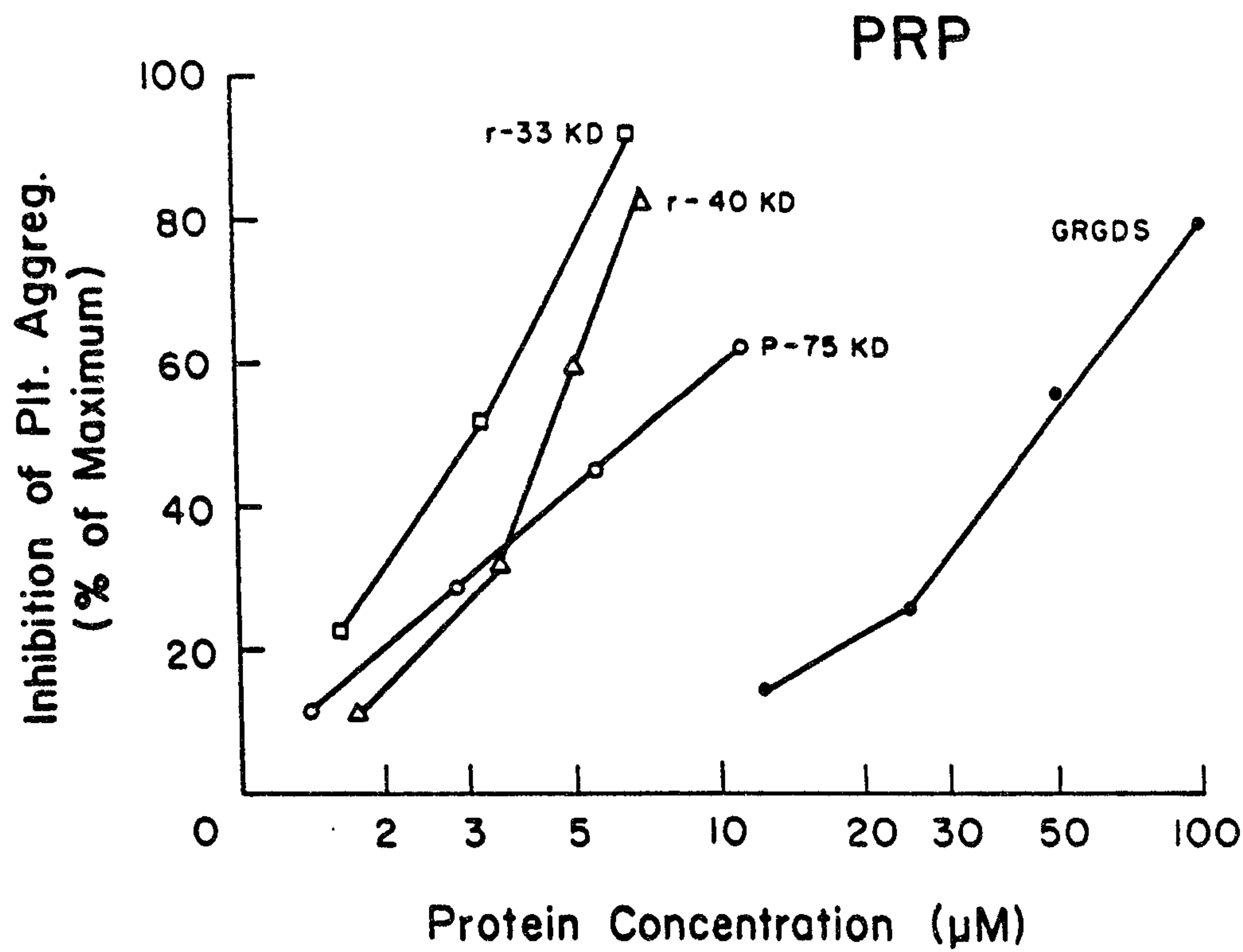
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Figure 27

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Figure 28

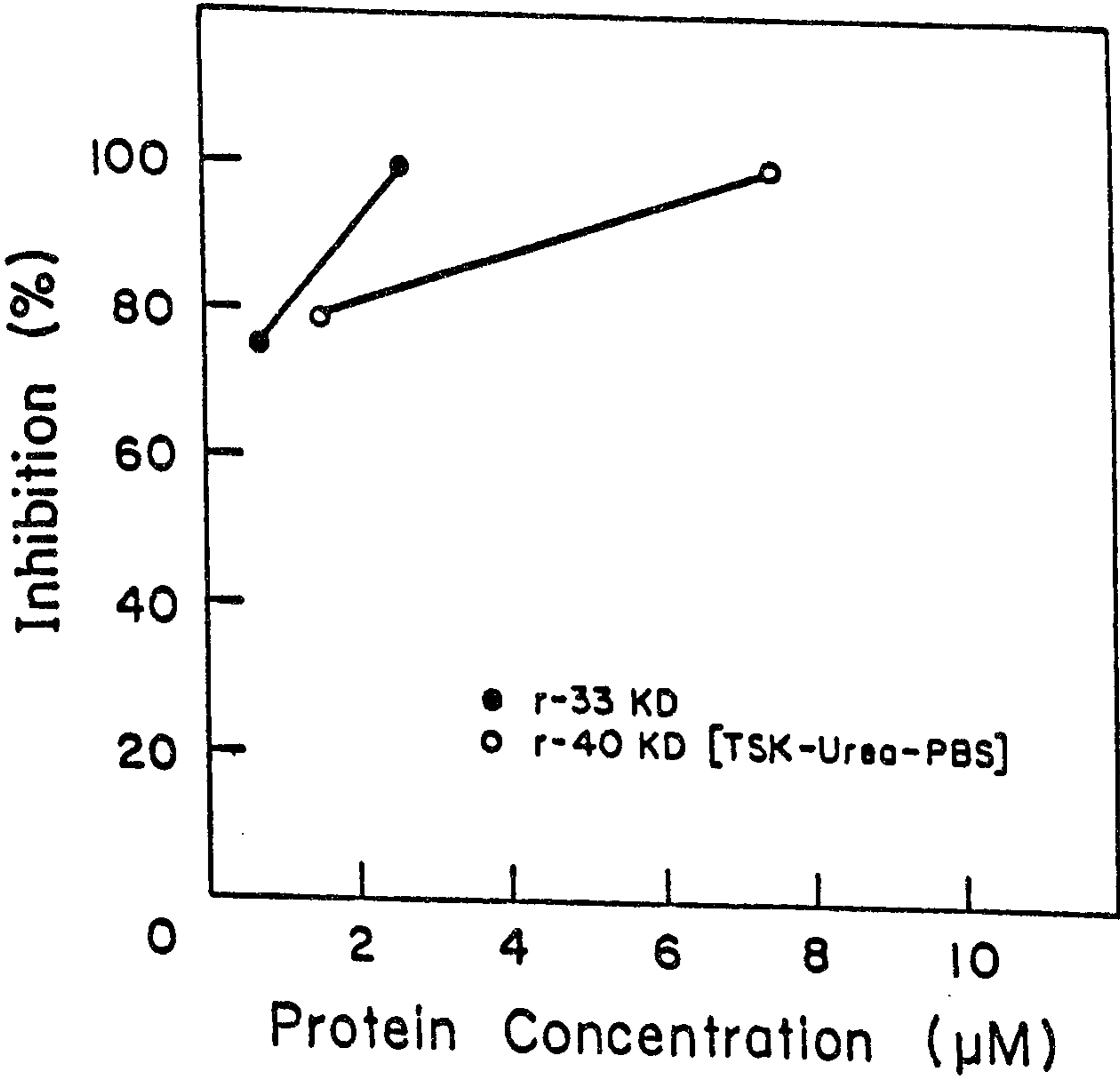


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Figure 29

Inhibition of Platelet Aggregation
in Whole Blood

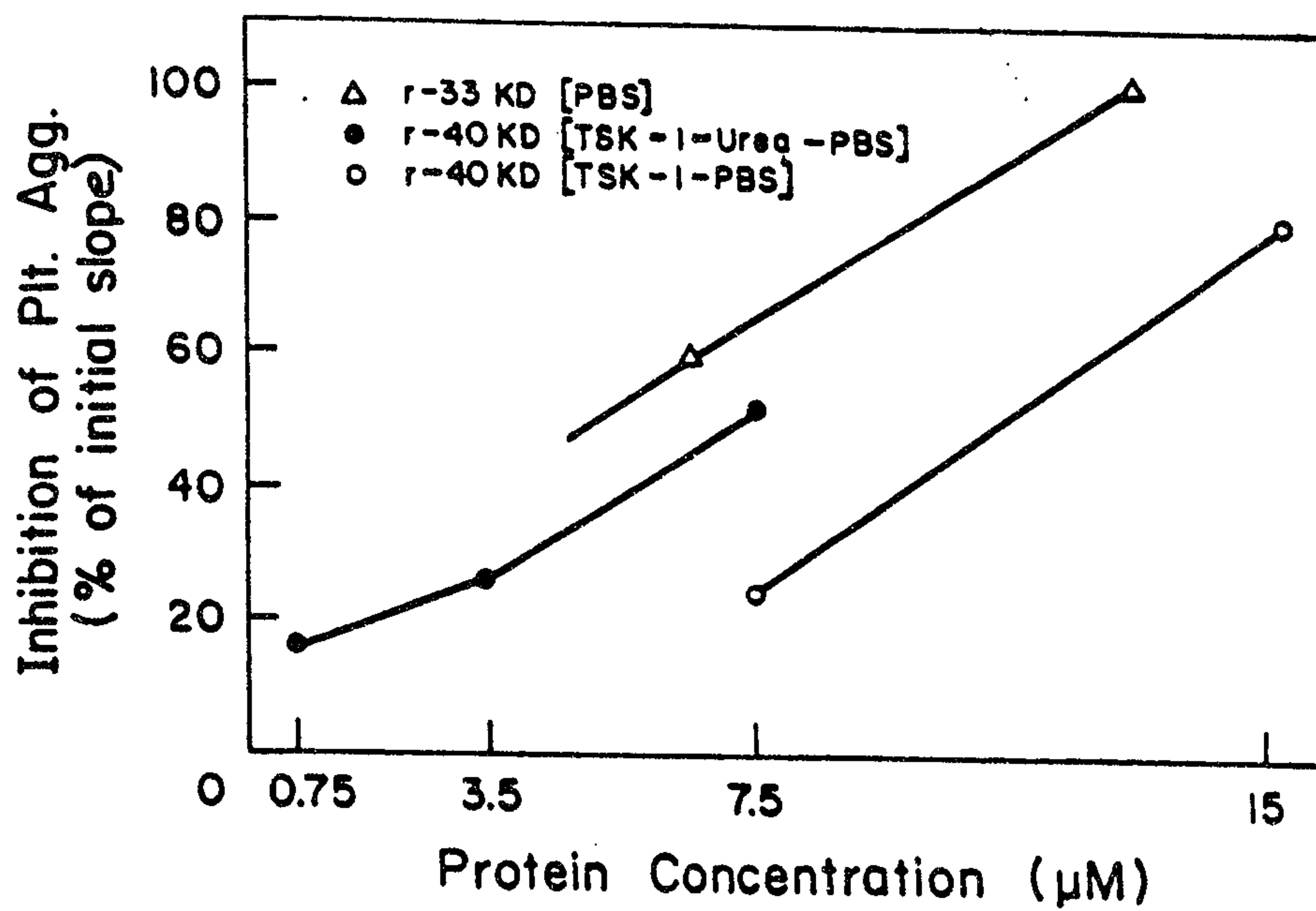


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Figure 30

Inhibition of Platelet Aggregation in PRP

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Figure 31

Binding of Adhesive Proteins to Platelets; Effect of Protein Concentration

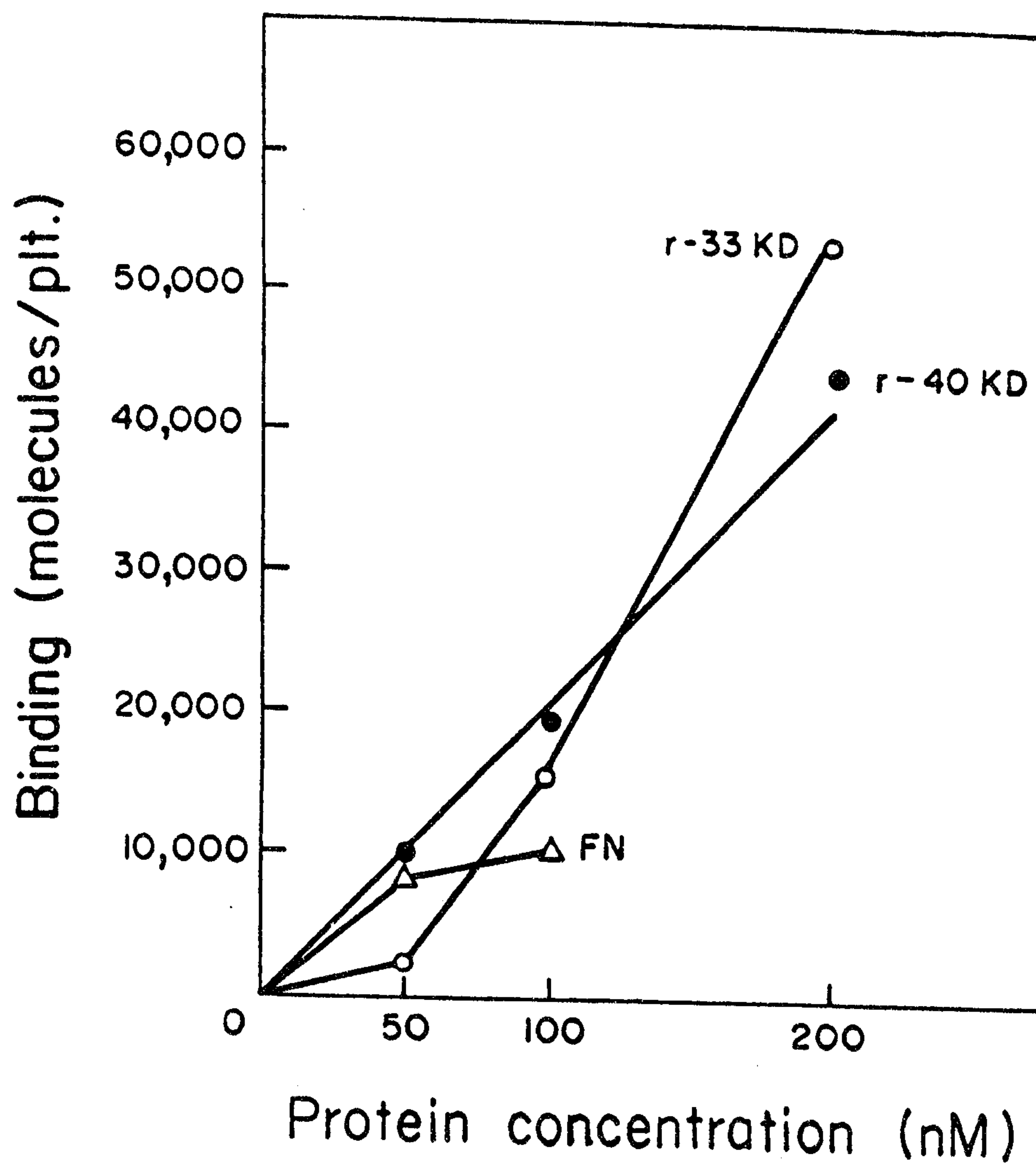
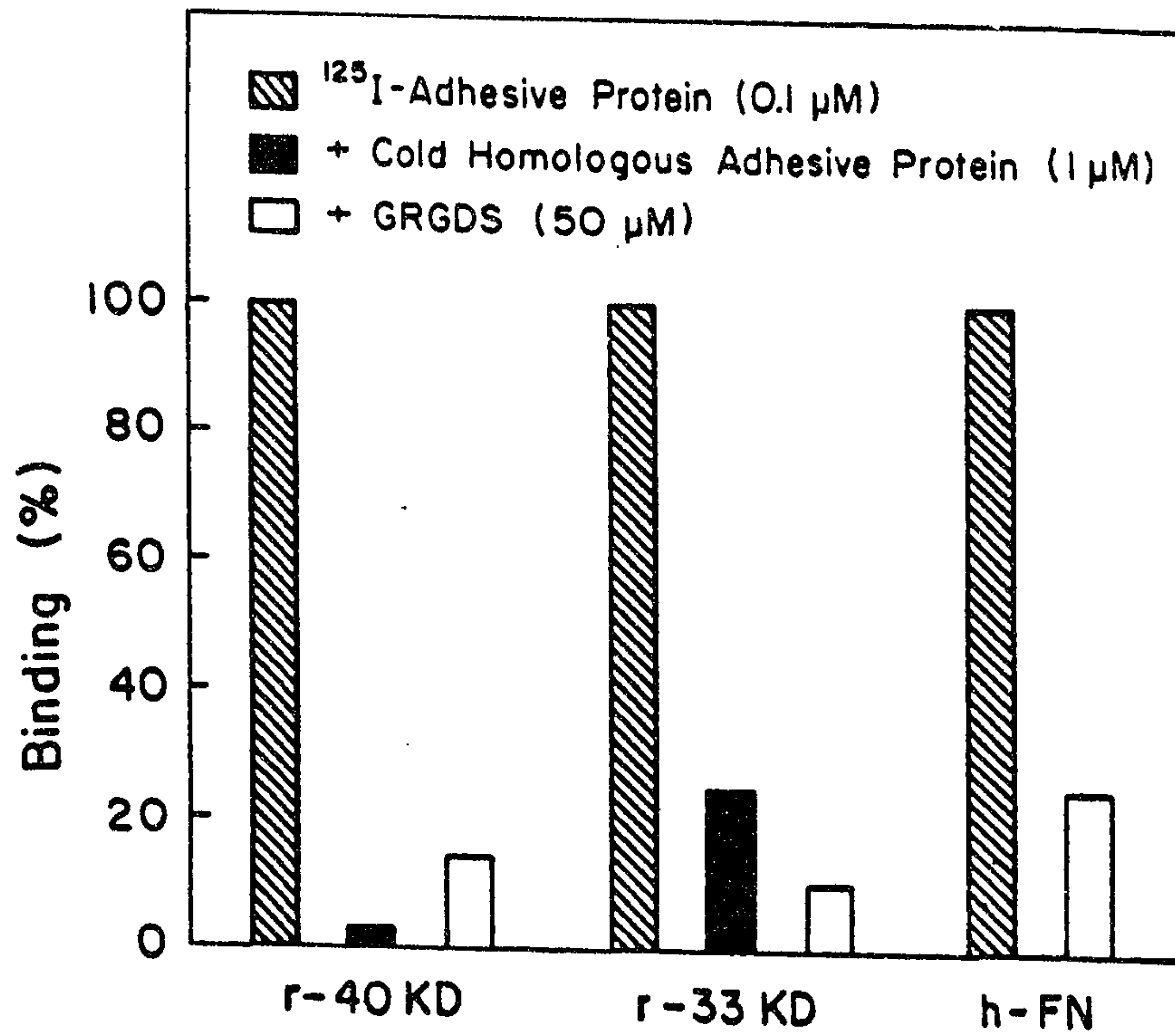
*Gowling, Strathy & Henderson*

Figure 32

Binding to Platelets of h-FN and
recombinant proteins; Effect of GRGDS



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Figure 33

Recombinant Adhesive Proteins Binding to Platelets
(100 nM ^{125} I protein; 5×10^8 Plt / ml)

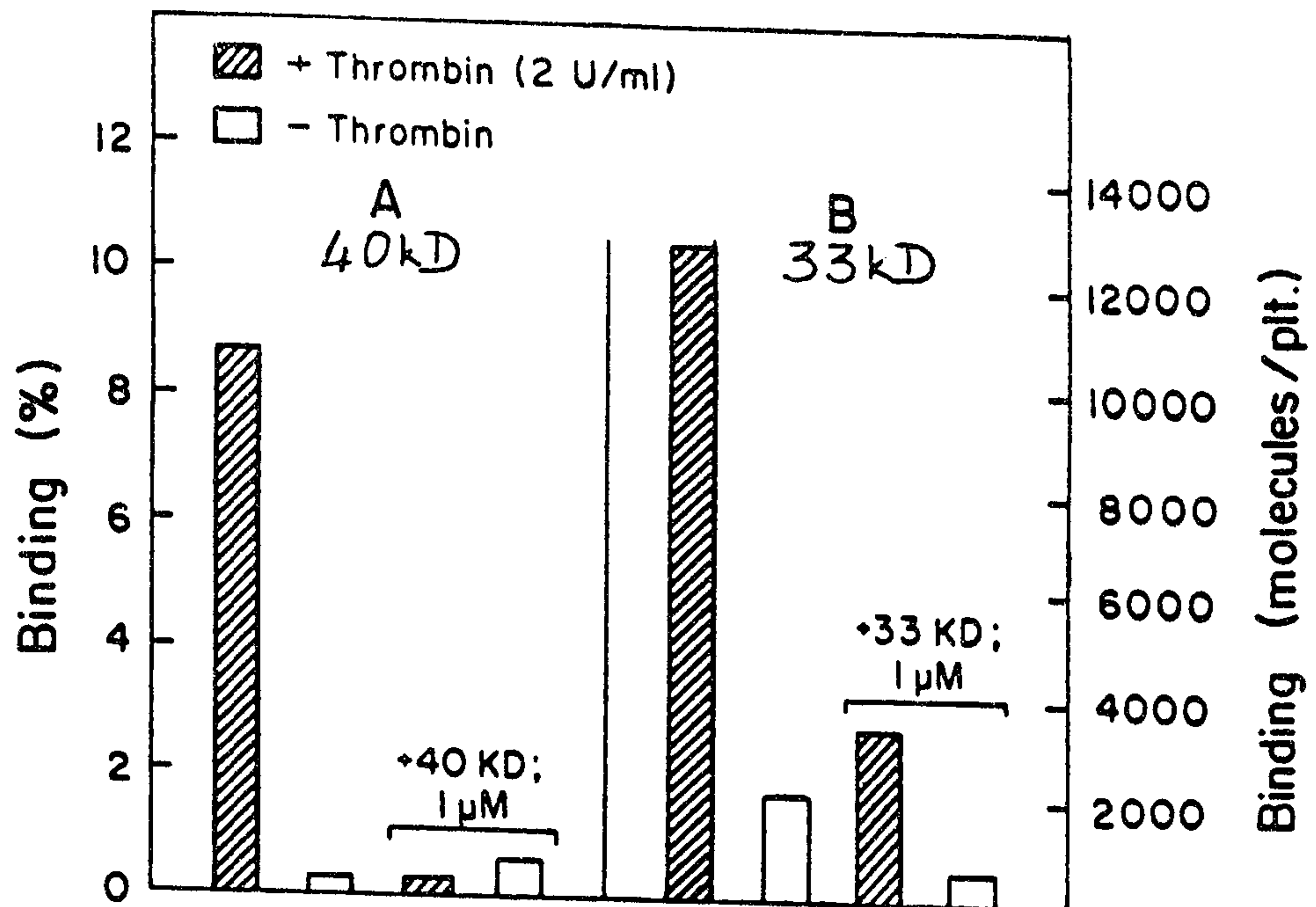
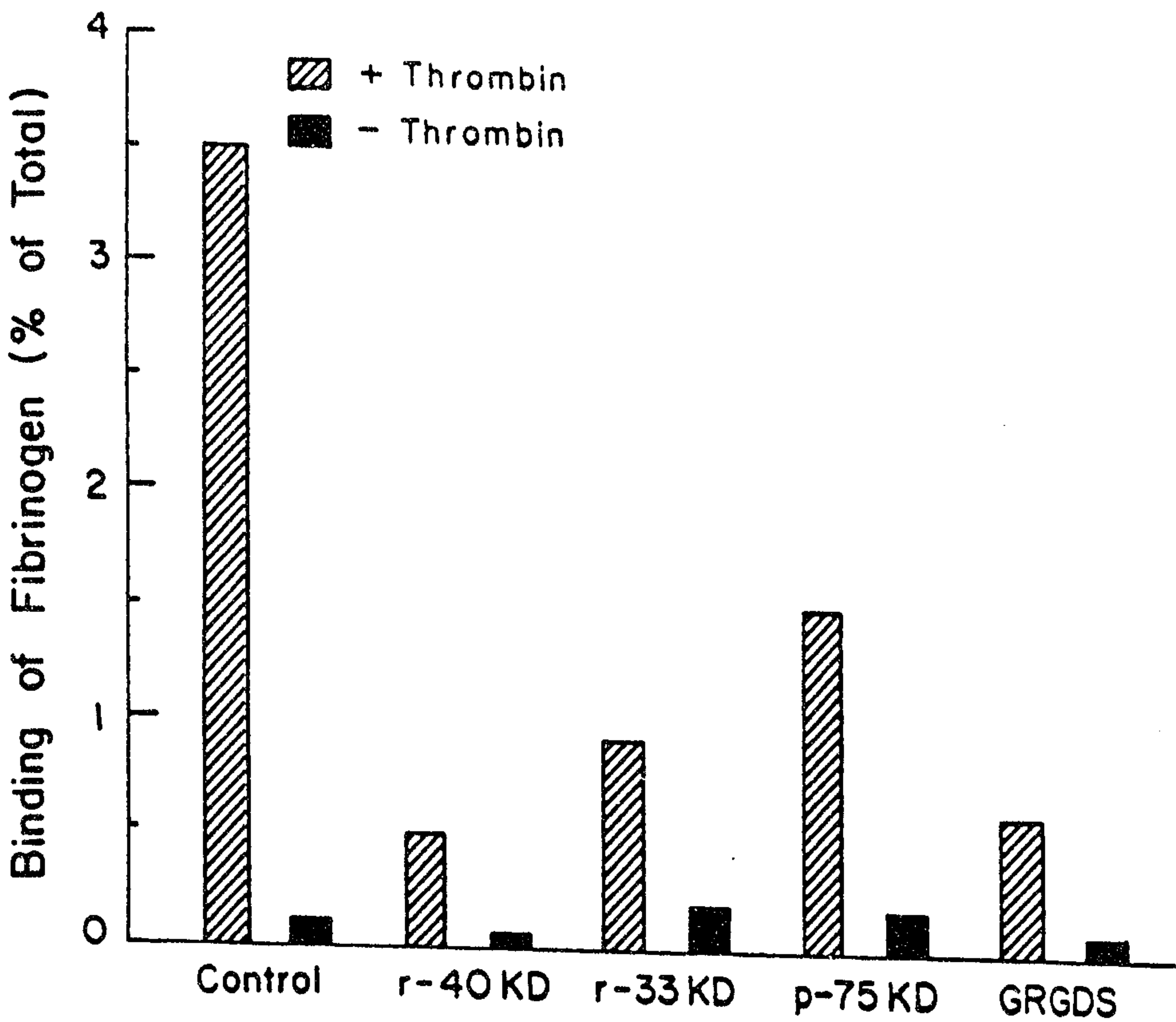
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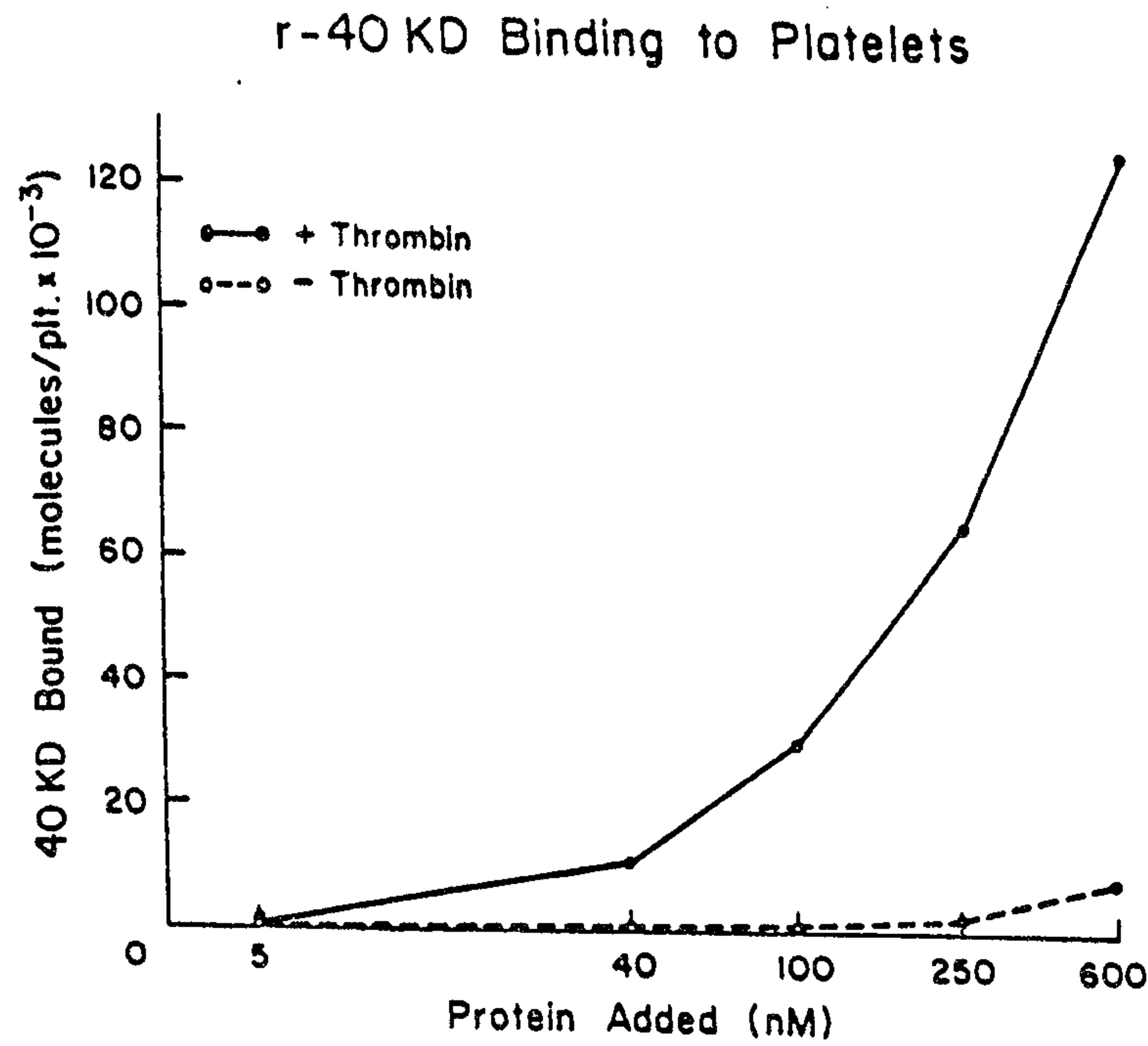
Figure 34

Binding of Fibrinogen to Platelets



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Figure 35A

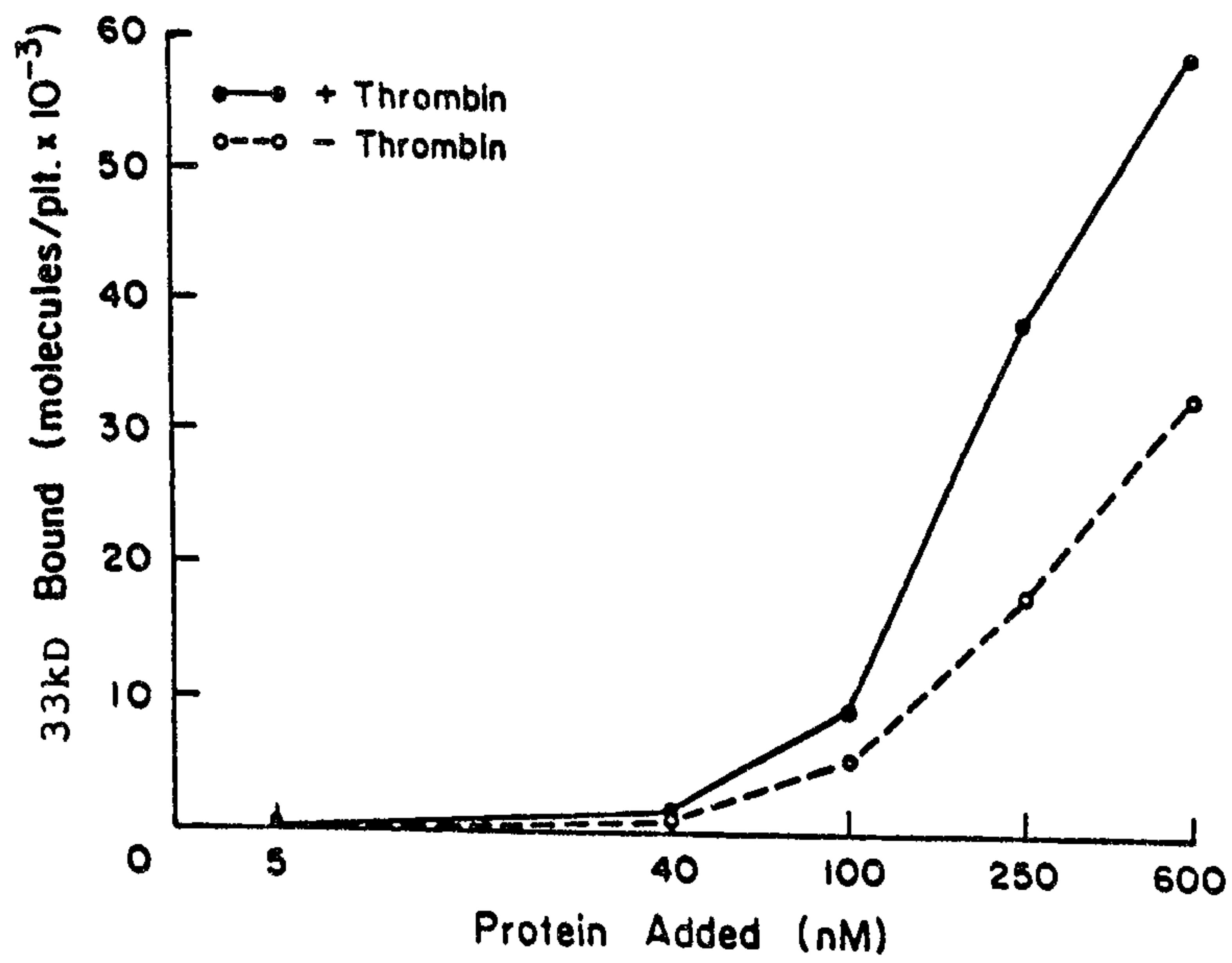


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Figure 35B

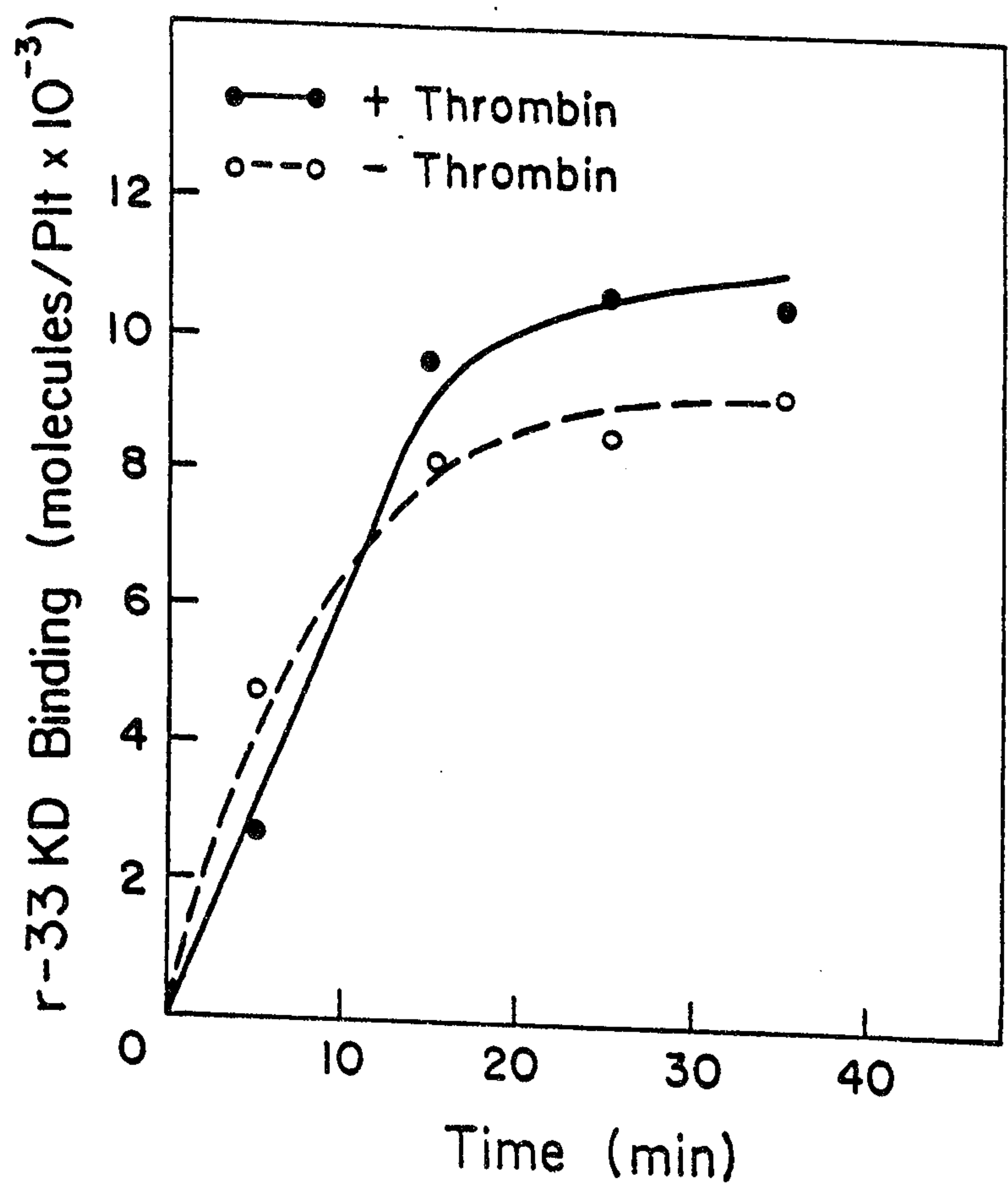
r-33 KD Binding to Platelets

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Figure 36

r-33 KD Binding to Plt; Effect of Thrombin and Incubation Time

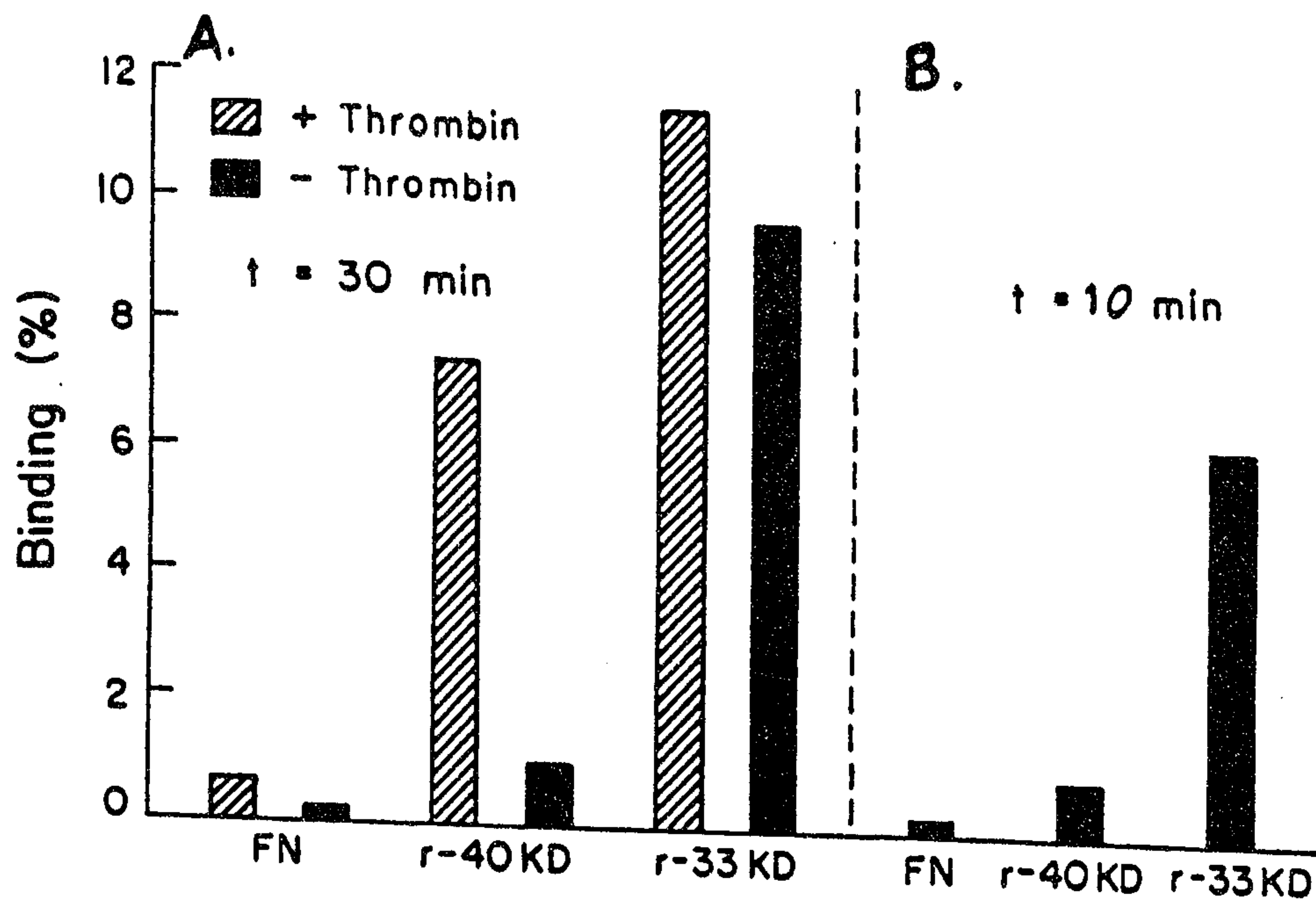


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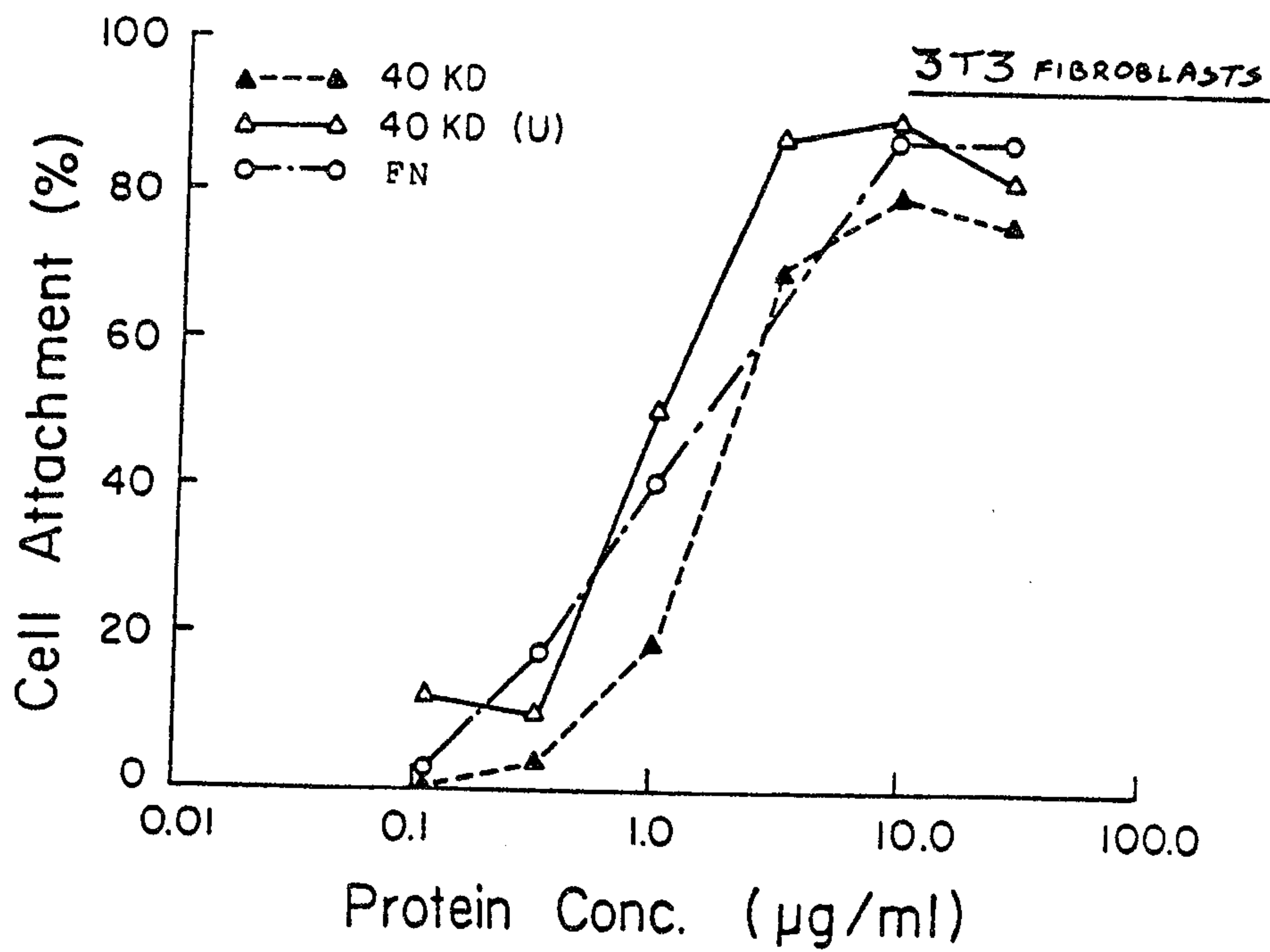
Figure 37

Adhesive Molecules Binding to Platelets

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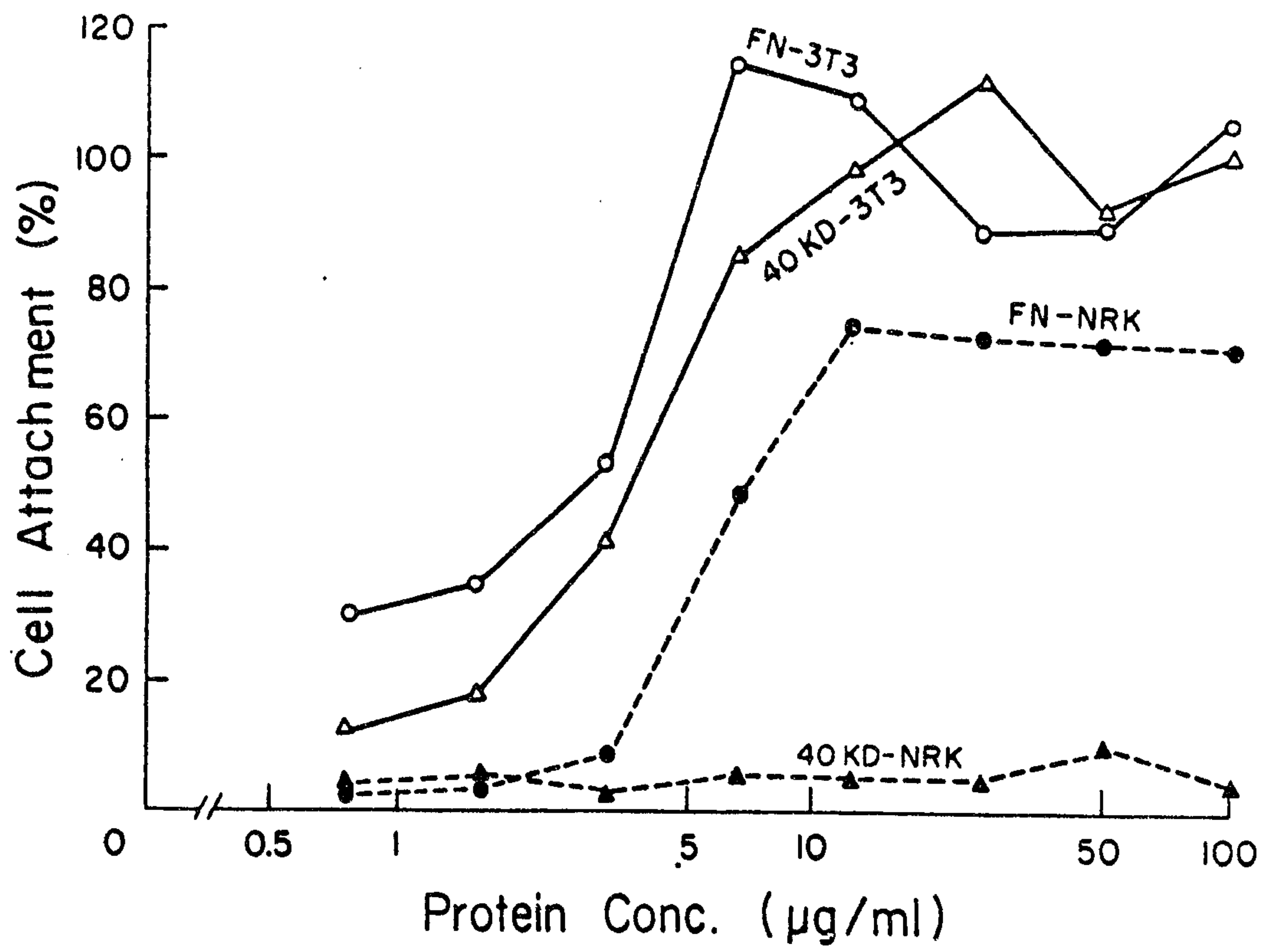
Figure 38A



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Figure 38B

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Figure 39

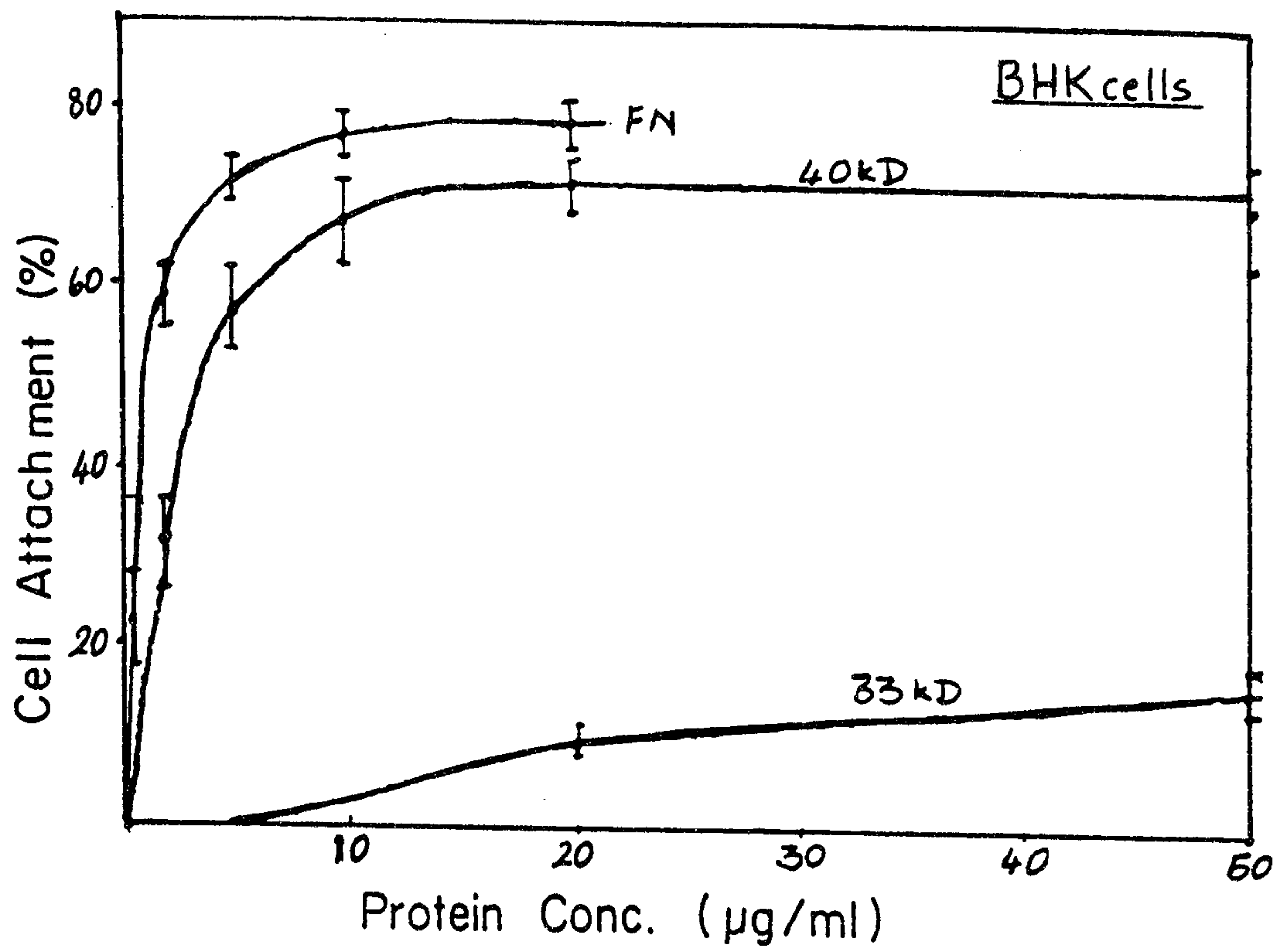
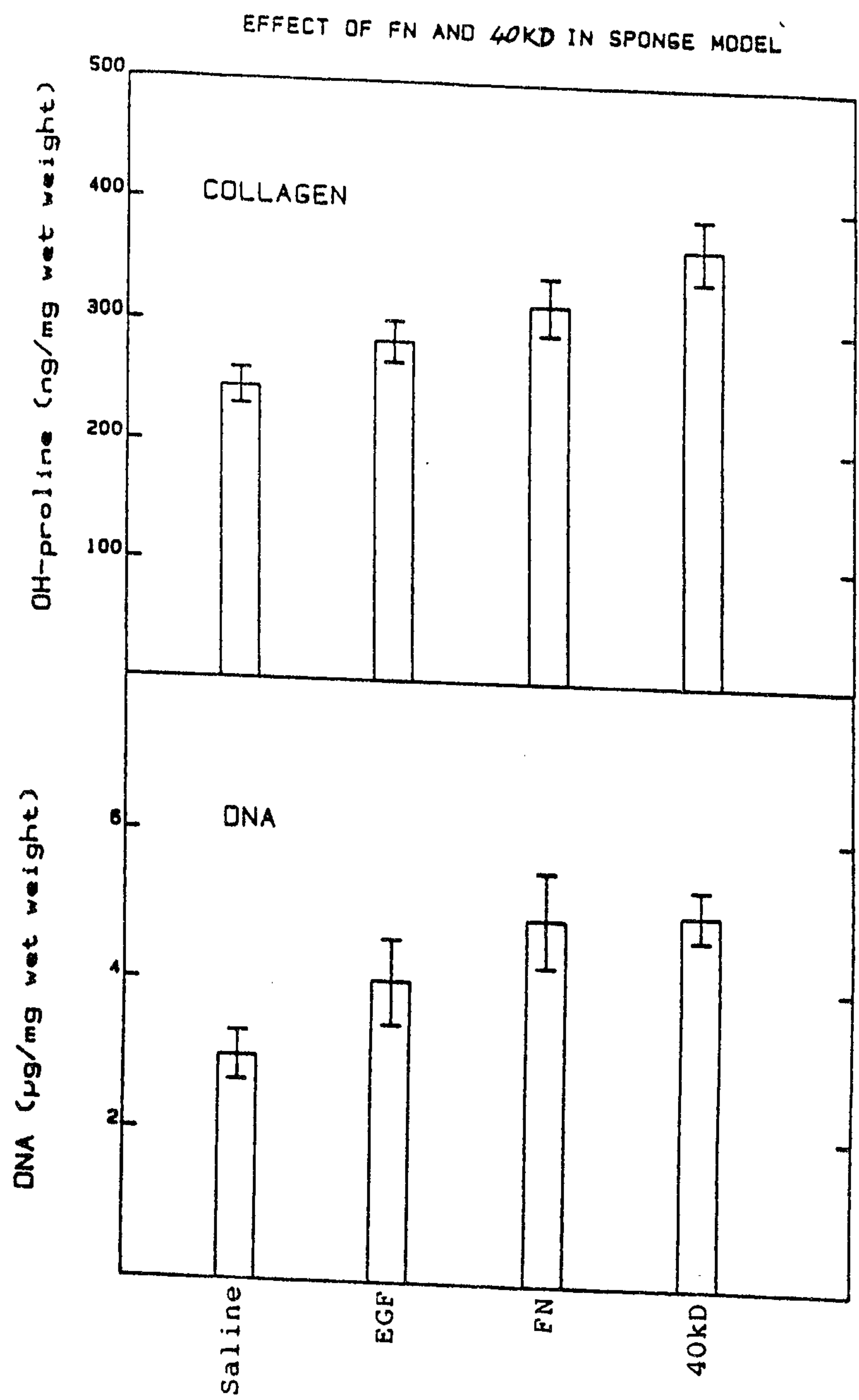
*Gowling, Strathy & Henderson*

Figure 40



Gowling, Strathy & Henderson

Figure 41B

T O T Y G G M L N G E P C V L P F T Y N G R T F Y S C T T E G R O D G H L W C S
A C C C A G A C T T A C G G T G G C A A C T T A A T G G A G A G C C A T G T G T T A C C A T T C A C C T A C A A T G G C A G G A G G T T C T A C T C C T G C A C C A G G A G G G G A C A G G A C G A C A T C T T T G G T E C A G
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080

T T S N Y E O D O K Y S F C T D H T V L V Q T O G G N S N G A L C H F P F L Y N
C A C A A C T T C G A A T T A T G A G C A G G A C C A G A A T A C T G T T T T G T T C A G A C T C A G G A G G A A T T C C A A T G G T G C C T T G T G C C A C T T O C C T T C C T A T A C A A
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200

N H N Y T D C T S E G R R D N M K W C G T T O N Y D A D O K F G F C P M A A H E
C A C C A C A A T T A C A C T G A T T G C A C T T C T G A G G G A G A G A C A C A C A T G A G T G G T G G G A C C A C A C A G A A C T A T G A T G O G A C C A G A A G T T T G G T T C T G C C C C A T G G C T G C C A C G A
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320

E I C T T N E G V M Y R I G D O W D K O H D M G H M M R C T C V G N G R G E W T
G G A A T C T G C A C A A C C A A T G A G G G T C A T G T A G G C A T T G G A T C A G T G G G A T A N C A G C A T G A C A T G G G T C A C A T G A T G A G T G C A C G T G T T G G A A T G G T G C T G G G A A T G G A C
1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440

C I A Y S O L R D O C I V D D I T Y N V M D T F M K R H E E G H M L N C T C F G
A T G C A T T G C C T A C T G G A C T T G C A G A T C A G T G C A T T G T T G A T G A C A C T T A C A A T G T G A C G A C A C A T T C C A C A G C G T C A T G A G G G G C A C A T G C T G A A C T G T A C A T G C T T C G G
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560

O G R G R W K C D P V D O C O D S E T G T F Y Q I G D S W E K Y V H G V R Y O C
T C A G G G T C G G C A G G T G G A A G T G T G A T C C G G T C G A C C A A T G C C A G G A T T C A G A G A C T G G G A C G T T T T A T C A A T T G G A G A T T C A T G G G A G A G T A T G T G C A T G G T G T C A G A T A C C A C T G
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680

Y C Y G R G I G E W H C O P L O Y Y P S S S G P V E V F I T E T P S O P N S H P
C T A C T G C T A T G G C G T G G C A T T G G G A G T G G C A T T G C C A A C C T T T A C A G A C C T A T C C A A G C T C A A G T G G T C T G C A A G T A T T A T A C T G A G A C T C C G A G T C A G O C C A A C T C C C A C C
1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800

I O W N A P O P S H I S K Y I L R W R P K N S V G R W K E A T I P G H L N S Y T
C A T C C A G T G G A A T G C A C C A G A C C A T C A C A T T C C A A G T A C A T T C T C A G G T G G A G A C C T A A A A T T C T G T A G C C G T T G G A G G A G A C T A C C A C A G G C C A C T T A A A C T C C T A C A C
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 2000

Figure 41c

I K G L K P G V V Y E G Q L I S I Q O Y G H Q E V T R F D F T T S T S T P V T
CATCAAGGCGCTGGTGGTATACGAGGGCCAGCTCATCAGCATCCAGCAAGTACGGCCAGCAAGTACGGCTTTCAGCTTCCAGCACCAGCAGCAGCAGCTGTGAC
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040

S N T V T G E T T P F S P L V A T S E S V T E I T A S S F V V S W V S A S D T V
CAGCAACACCGTGCAGGAGAGAGAGAGCTCCCTTTCTCTCTGTTGGCCACTTCTGAATCTGGAOCCGAATCAGCCAGTAGCTTTGGTCTCTGGTCTCAGCTTCCGACACCGT
2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160

S G F R V E Y E L S E E G D E P Q Y L D L P S T A T S V N I P D L L P G R K Y I
GTGGGATTCCGGTGGGAATATGAGCTGAGTGAGGAGGAGATGAGCCAGTACCTGGATCTTCCAGCAGCAGCCACTTCTGTGAACATCCCTGAOCTGCTTCTCTGCGCCAAATACAT
2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280

V N V Y Q I S E D G E Q S L I L S T S Q T T A P D A P P D P T V D Q V D D T S I
TGTAATGTCTATCAGATATCTGAGGATGGGAGCAGAGTTGATCCTGTCTACTTCACAAACACAGGCGCTGATGCGCTCTGACCGACTGTGGACCAAGTTGATGACACCTCAAT
2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400

V V R W S R P Q A P I T G Y R I V Y S P S V E G S S T E L M L P E T A K S V T L
TGTTGTTGCTGGAGCAGACGCCAGGCTCCCATCAGGGTACAGATAGTCTATTGCGCATCAGTAGAGGTAGCAGCAGACAGACTCAAGCTTCTGTAAGTCAAACTCCGTCACCGT
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520

S D L Q P G V Q Y N I T I Y A V E E N Q E S T P V V I Q Q E T T G T P R S D T V
CAGTGACTTGCAOCTGGTTCAGTATACATCACTATCTATGCTGTGGAGAAATCAGAAAGTACAOCTGTGTTCATTCACAGAAACCACTGCGCAOCCCAOCCGCTCAGATACAGT
2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640

P S P R D L Q F V E V T D V K V T I M N T P P E S A V T G Y R V D V I P V N L P
GCGCTCTCCAGGACCTGCAGTTTGTGGAAGTGACAGAGCTGAAGTCACCATCATGTGGACAACCGCTGAGAGTGCAGTACCGCTACCGTGGATGTGATCCCGCTCAACCTGCG
2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760

G E H G Q R L P I S R N T F A E V T G L S P G V T Y Y F K V F A V S H G R E S K
TGCGGAGCAGCGGCGAGAGGCTGGCCATCAGCAGCAACCTTTGCAAGAGTCACCGGCTGTCOCCCTGGGCTCACCTTACTTCAAGTCTTTCAGTACGCCATGGGAGGAGAGCAA
2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880

Figure 41D

19
 P L T A Q Q T T K L D A P T N L O F V N E T O S T V L V R W T P P R A Q I T G Y
 2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
 G C T C T G A C T C A C A G A C A A C A A C T G G A T G C T C A C T A C C T G C A T T G T C A A T G A A C T G A T T C T A C T G T G T G T G A T G S A C T C C A C C T C G G C C C A G A T A A C A G G A T A
 R L T Y G L T R R G Q P R Q Y N V G P S V S K Y P L R N L Q P A S E Y T V S L V
 3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120
 C C G A C T G A C G T G G G C T T A C C G A A G A G C A G C C A G C C A G C A G T A C A T G T G G G T C C T C T G T C A A G T A C C C C T G A G G A A T C T G A C G C T C A T C T G A G T A C A C C G T A T C C C T C G T
 A I K G N Q E S P K A T G V F T T L Q P G S S I P P Y N T E Y T E T T I V I T W
 3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240
 G G C C A T A A G G C C A C C A A G A G A G G C C C A A G C C A C T G G A G T C T T T A C C A C A C T G C A G C C T T G G S A G C T C T A T T C C A C C T T A C A C A C C G A G T G A C T G A G A G C A C C A T C G T G A T C A C A T G
 T P A P R I G F K L G V R P S Q G G E A P R E V T S D S G S I V V S G L T P G V
 3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360
 G A C G C C T G C C A G A A T T G T T T A A G C T G G G T G A G A C C A A G C C A G G A G G A G C A C C A G A G A G T G A C T T C A G A C T C A G A A G C A T C G T T G T G C C G C T T G A C T C A G G A G T
 E Y V Y T I Q V L R O G O E R O A P I V N K V V T P L S P P T M L H L E A N P D
 3370 3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480
 A G A T A G C T C T A C A C C A T C C A A G T C C T G A G A G A T G G A C A G A A G A G A T G G C C A T T G T A A C A A A G T G G T G A C A C C A T T G T C T C C A C C A C A A A C T T G C A T C T C G A G C A A A C C C T G A
 T G V L T V S W E R S T T P D I T G Y R I T T Y P T N G O O G N S L E E V V H A
 3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
 C A C T G G A G T G C T C A C A G T C T C T G G A G A G G A G C A C C A C C A C A C A T T A G A A T T A C C A C A C C C T A C A A C G C C A G C A G G A A T T C T T T G A A G A A G T G G T C A T C C
 D O S S C Y F D N L S P G L E Y N V S V Y T V K D D K E S V P I S D T I I P A V
 3610 3620 3630 3640 3650 3660 3670 3680 3690 3700 3710 3720
 G G A T C A G A G C T C T G C A C T T T T G A T A C C T G A G T C C G G C T G G A G T A C A A T G T C A G T T T A C A C T G T C A A G G A T G A C A A G A A G T G C C T A T C T C T G A T A C C A T C A T C C C A G C T G T
 P P P T D L R F T N I G P O T M R V T W A P P P S I D L T N F L V R Y S P V K N
 3730 3740 3750 3760 3770 3780 3790 3800 3810 3820 3830 3840
 T C C T C T C C A C T G A C C T G G G A T T C A C C A C A T T G T C C A G A C A C C A T G C G T G T C A C T G G C C C A C C C A T C C A T T G A T T A A C C A A C T T C C T G G T G C T T A C T C A C C T G T C A A A A

E E D V A E L S I S P S D N A V V L Y N L L P G T E Y V V S V S S Y E Q M E S
TGAGGAAGATGTTGCAGAGTGTCAATTCTCCTTCAGACAAATCCCTGGCTGGTACAGAATATGTAGTAGTGTCTCAGTGTCTAOGAACAACATGAGAG
3850 3860 3870 3880 3890 3900 3910 3920 3930 3940 3950 3960

T P L R G R O K T G L D S P T G I D F S D I T A N S F T V H W I A P R A T I T G
CACAOCTCTTAGAGGAACAGAAACAGGCTTGATTOCCAACIGSCATGACTTTTCIGATATTACTGCCAACICTTTIACITGTGCACITGGATTGCTCCTCGAGCCACCATCACTGG
3970 3980 3990 4000 4010 4020 4030 4040 4050 4060 4070 4080

Y R I R H H P E H F S G R P R E D R V P H S R N S I T L T M L T P G T E Y V V S
CTACAGGATCGGOCATCATOCCGAGCACCTTCAGTGGGAGATGGGTGCCACTCTCGGNATTCACCCCTCAACCCTCACTCCAGGCACAGAGTATGTGTCAG
4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200

I V A L N G R E E S P L L I G O Q S T V S D V P R D L E V Y A A T P T S L L I S
CATOGTTGCTCTTAATGACAGAGAGGAAGTCCCTATTGATTGGCCAAACATCAACAGTTTCTGATGTTCCGAGGCACTTGGAAAGTTGTTCTGCGAACCACCAGCCTACTGATCAG
4210 4220 4230 4240 4250 4260 4270 4280 4290 4300 4310 4320

W D A P A V T V R Y R I T Y G E T G G N S P V Q E F T V P G S K S T A T I S G
CTGGGATGCTCCTCCTGTCACAGTGAGATATTACAGGATCACTTACGAGAAACAGGAGGAATAAGOODTGTCCAGGAGTTCACTGTGCTGGAGCAAGTCTACAGCTACCATCAGCGG
4330 4340 4350 4360 4370 4380 4390 4400 4410 4420 4430 4440

L K P G V D Y T I T V Y A V T G R G D S P A S S K P I S I N Y R T E I D K P S Q
OCTTAAOCTGGAGTTGATTATACCATCACTGTGTATGCTGTCACTGGCGTGAGACAGCOOCCGANGCAGCAAGCCAAATTCOAATTAATACCGAACAGAAATTCACAACCATCCCA
4450 4460 4470 4480 4490 4500 4510 4520 4530 4540 4550 4560

M Q V T D V Q D N S I S V K W L P S S P V T G Y R V T T T P K N G P G P T K T
GATGCAAGTGAOCCGATGTTACAGCAACAGCATTAGTGTCAMGTGCTGCTTCAAGTTCCCTGTTACTGGTTACAGAGTAACCCACTCCCAAATGGACCAAGCAAAAAC
4570 4580 4590 4600 4610 4620 4630 4640 4650 4660 4670 4680

K T A G P D Q Q T E M T I E G L Q P T V E Y V S V Y A Q N P S G E S Q P L V Q T
TAAACTGCAGTCCAGATCAACAGAAATGACTATTGAAGCCTTGACGCCACAGTGGAGTATGGTTAGTGTCTATGCTCAGAAATCCAGGAGAGAGTGCAGCCTCTGTTTCAGAC
4690 4700 4710 4720 4730 4740 4750 4760 4770 4780 4790 4800

Figure 41F

26
A V T N I D R P K G L A F T D V D S I K I A W E S P Q G Q V S R Y R V T Y S
TGCAGTAAACCAACATTGATCGCCCTAAAGGACTGGCATTCACTGATGGATGGATTCATCAAAATTGCTTGGGAAGGCCACAGGGGCAAGTTTCCAGGTACAGGGTGACCTACTC
4810 4820 4830 4840 4850 4860 4870 4880 4890 4900 4910 4920
S P E D G I H E L F P A P D G E E D T A E L Q G L R P G S E Y T V S V V A L H D
GAGCCCTGAGGATGGAAATCCATGAGCTATTCCCTGCACCTGATGGTGAAGAGACACTGCAGAGCTGCAAGCCCTCAGAGCCGGTTCTCAGTACACAGTCAGTGTGGTTGCCCTTGCACGA
4930 4940 4950 4960 4970 4980 4990 5000 5010 5020 5030 5040
D H E S Q P L I G T O S T A I P A P T D L K F T Q V T P T S L S A Q M T P P N V
TGATATGGAGAGCCAGCCCTGATTGGAAOCCAGTATTCCTGCACCACTGACCTGAAGTTCACTCAGGTCAACCCACAGCCCTGAGGCCAGTGGACACCCACCCAAATGT
5050 5060 5070 5080 5090 5100 5110 5120 5130 5140 5150 5160
Q L T G Y R V T P K E K T G P M K E I N L A P D S S S V V V S G L M V A T K
TCAGCTCACTGGATATCGAGTGGGGTGACCCCAAGGAGAGACCGGACCAATGAAGMAATCAOCTTCTCTCTCAGCTCATCGGTGGTTGTATCAGGACTTATGGTGGCCACCAA
5170 5180 5190 5200 5210 5220 5230 5240 5250 5260 5270 5280
Y E V S V Y A L K D T L T S R P A Q G V V T Y T L E N V S P P R R A R V T D A T E
ATATGAAGTGAGTGTCTATGCTCTTAAGGACACTTTGACAGGAGACAGCTCAGGGTGTGTCAOCCACTCTGGAGAAITGTAGGCCCAACCAAGAGGCCCTGCTGTGACAGATGCTACTGA
5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400
T T I T I S W R T K T E T I Y G F Q V D A V P A N G Q T P I Q R T I K P D V R S
GAOCCATCACCATTAGCTGGAGAACCAAGACTGAGAGCATCACTGGCTTCCAGTTGATGGCTTCCAGCCAAATGGCCAGACTCCAAATCCAGAGAACCAATCAAGCCAGATGTCAGNAG
5410 5420 5430 5440 5450 5460 5470 5480 5490 5500 5510 5520
Y T I T G L Q P G T D Y K I Y L Y T L M D N A R S S P V V I D A S T A I D A P S
CTACACCATCAGGGTTTACAAOCCAGGCACTGACTACAGATCTAOCCTGTACACCTTGAATGACAAATGCTCGAGGCTCCCTGTGGTCAATGAGGCCCTCCACTTCCATTGATGCCACATC
5530 5540 5550 5560 5570 5580 5590 5600 5610 5620 5630 5640
N L R F L A T T P N S L L V S W Q P P R A R I T G Y I I K Y E K P G S P P R E V
CAOCTGGGTTTCTGGCCAGCACACCAOCCAAATTCCTTGGTATCATGTCAGCGGCCAGCTGCCAGGATTACGGGTACATCATCAAGTATGAGAGCCCTGGGTGCTCTCCACAGAGAGT
5650 5660 5670 5680 5690 5700 5710 5720 5730 5740 5750 5760

Figure 41G

V P R P R P G V T E A T I T G L E P G T E Y T I Y V I A L K N M Q K S E P L I G
GGTCCCTCGGCCCCCCTGGTGTACAGAGGCTACTATTACTGGCTGGAAOCGGAAOCGAAATATACAAATTTATGTCATTC-CCCTGAAGATAATCAGAAGAGGAGCCCTGATTGG
5770 5780 5790 5800 5810 5820 5830 5840 5850 5860 5870 5880

³⁰
R K K T D E L P Q L V T L P H P N L H G P E I L D V P S T V Q K T P F Y T H P G
AAGGAAAGACAGACAGAGCTTCCCAACTGGTAACCTTCCACACCCCAATCTTCATGGACACAGAGATCTTGGATGTTCTTCCACAGTTCCAAAGAACCCCTTTCGTACCCACCCCTGG
5890 5900 5910 5920 5930 5940 5950 5960 5970 5980 5990 6000

Y D T G N G I Q L P G T S G Q Q P S V G Q Q M I F E E H G F R R T T P T I A T
GTATGACACTGGAAATGGTATTGAGCTTCTGCTGAGCAACCCAGTGTGGCAACAATGATCTTTGAGGAACATGGTTTACGGCGGACCAACCCCTTTCGTACCCACCCCTGG
6010 6020 6030 6040 6050 6060 6070 6080 6090 6100 6110 6120

³¹
P I R H R P R P Y P P N V G Q E A L S Q T T I S W A P F Q D T S E Y I I S C H P
CCCATAGGCATAGGCCAAGACCATACCCCGAATGTAGGACAGAGCTCTCTCAGACACACCATCTGCTGAGCAACATTCCTGAGTACATCATTTGATGTCATCC
6130 6140 6150 6160 6170 6180 6190 6200 6210 6220 6230 6240

Y G T D E E P L Q F R V P G T S T S A T L T G L T R G A T Y M I I V E A L K D Q
TGTTGCCACTGATGAGAACCTTACAGTTGAGGTTCCCTGGAATCTCTACAGTGGCACTCTGACAGGCTGACAGAGGTCACCTACACATCATAGTGGAGGCACTGAAGACCA
6250 6260 6270 6280 6290 6300 6310 6320 6330 6340 6350 6360

³²
Q R H K V R E E V V T V G N S V N E G L N Q P T D D S C F D P Y T V S H Y A V G
GCAGAGGCATAAGTTGGGAGAGGTTGTACCGTGGCAACTCTGTCAAGCAGGCTTGAACCAAGCTAGGATGCTGCTTTCACCCCTACACAGTTTCCATTATGCCGTTGG
6370 6380 6390 6400 6410 6420 6430 6440 6450 6460 6470 6480

³³
D E W E R M S E S G F K L L C Q L L G F G S G H F R C D S S R W C H D N G V N Y
AGATGAGTGGGAACGAATGTCTGAATCAGGCTTTAACTGTGTGCCAGTGTCTTAGGCTTTGGAAAGTGGTCAATTCAGATGTGATTCATCTAGATGTCACAAATGCTGTCACCTA
6490 6500 6510 6520 6530 6540 6550 6560 6570 6580 6590 6600

³⁴
K I G E K W D R Q G E N G Q M M S C T C L G N G K G E F K C D P H E A T C Y D D
CAAGATTGGAGAGTGGCAACCTCAGGAGAAATGGCAGATGAGCTGCACATGTCTTGGGACGGAAGAGAGATTCAGTGTGACCCCTCATGAGGCAACGCTGTACGATGA
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700 6710 6720

³⁵

Figure 41H

G K T Y H V G E Q W Q K E Y L G A I C S C T C F G G Q R G V R C D M C R R P G 36
TGGAGACATACACGTAGGAGAACAGTGGCAGAGAGGAATATCTGGGTGCCATTGCTCTGTCACATGCTTTGGAGGCCACGGGGGCTGGCCCTGTGACAACTGGCCGACAGACCTGGGG
6730 6740 6750 6760 6770 6780 6790 6800 6810 6820 6830 6840

E P S P E G T T G Q S Y N Q Y S Q R Y H Q R Y N T N V N C P I E C F M P L D V Q
TGAACCCAGTCCCGAAGGCCACTACTGGCCAGTCCCTACACCCAGTATCTCAGAGATAOCCATCAGAACACACACTAATGTTAATGCCCAATTGAGTGTCTTCATGCCCTTTAGATGTACA
6850 6860 6870 6880 6890 6900 6910 6920 6930 6940 6950 6960

A D R E D S R E
GGCTGACAGAGAGATTCCCGAGAGTAATCATCTTTCCCAATCCAGAGGACACAGCATGTCTCTGCCAAGATCCATCTAACTGGAGTGAATGTIAGCAGACCCAGCTTAGAGTTCTTC
6970 6980 6990 7000 7010 7020 7030 7040 7050 7060 7070 7080

TTTCTTTCTTAAGCCCTTGTCTCTGGAGGAAGTCTCCAGCTTCAGCTCACTCACAGCTTCTCCAAGCATCACCCCTGGAGTTTCTCTAGGGGTTTCTCATAAATGAGGGCTGCACATT
7090 7100 7110 7120 7130 7140 7150 7160 7170 7180 7190 7200

GCTGTCTGCTTCGAGTATTCGAATACGGCTCAGTATTTTAAATGAGTGATTTCTAAGATTTGGTTTGGGATCAATAGGAAGCATATGCCAGCCACCAAGATGCCAAATGTTTGAAT
7210 7220 7230 7240 7250 7260 7270 7280 7290 7300 7310 7320

GATATGACCAAAATTTTAAGTAGGAAGTCAOCCAAACACTCTGCTTTCAGTTAAGTGTCTGGCCCAATACGTAGGACACAGCAATGATCTTGTIACGTGTGATATTTTAAATATCCA
7330 7340 7350 7360 7370 7380 7390 7400 7410 7420 7430 7440

CAGTACTCACTTTTTCCAATGATCCTAGTAATTGCTAGAAATATCTTCTCTTAOCTGTATTTATCAATTTTTCOCAGTATTTTATACGGAAAAATGTATTGAAAACACTTAGT
7450 7460 7470 7480 7490 7500 7510 7520 7530 7540 7550 7560

ATGCAGTTGATAGAGGAATTTGGTATAATTAATGGTGGTGATTATTTTATACGTGATGTGCCAAGCTTTIACIACGTGTGGAAGACAACTGTTTAAATAAAGATTIACATTCACACA
7570 7580 7590 7600 7610 7620 7630 7640 7650 7660 7670 7680

AAAAAAAAAAAAAAAAAAAA
7690 7700 5 15 25 35 45 55 65 75 85 95

Figure 42A

Pair 1
1 5'- AATTCAATATGCAGGC^{*}ACAGCAAATGGTTTCAGCCCCAGTCCCCGGTGGCTGTCAAGTCAAGCAAGCCCGGT - 3'
2 3'- GIATACGTCCTCGTGTGTTTACCAAGTCGGGTGAGGGGCCACCGACAGTCAGTTTCGTTGGGCCAACAAATA - 5'

Pair 2
3 5'- GTTATGACAATGGAAACACTATCAGATAAATCAACAGTGGGAGCGGACCTACCTAGGTAATGTGTG - 3'
4 3'- CTGTTACCTTTTGTGATAGTCTATTTAGTTGTCACCCCTCGCCTGGATGGATCCATT - 5'

Pair 3
5 5'- GTTTGACTTGTTATGGAGGAGCCGAGGTTTTAACTGCGAAAGTAAACCTGAAGCT - 3'
6 ACACAACCAACATGAACAATACCTCCTTCGGTCCAAAATTGACGCTTTCATTTGGACTTCGACTTCTCT - 5'

Pair 4
7 5'- GAAGAGACTTGCTTTGACAAGTACACTGGGAACACTTACCGAGTGGGTGACACTTATGAGCGTCCCTAAA - 3'
8 3'- GAACGAAACTGTTTCATGTGACCCCTTGTGAATGGCTCACCCCACTGTGAATACTCGCAG - 5'

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Gowling, Strathy & Henderson

2006929

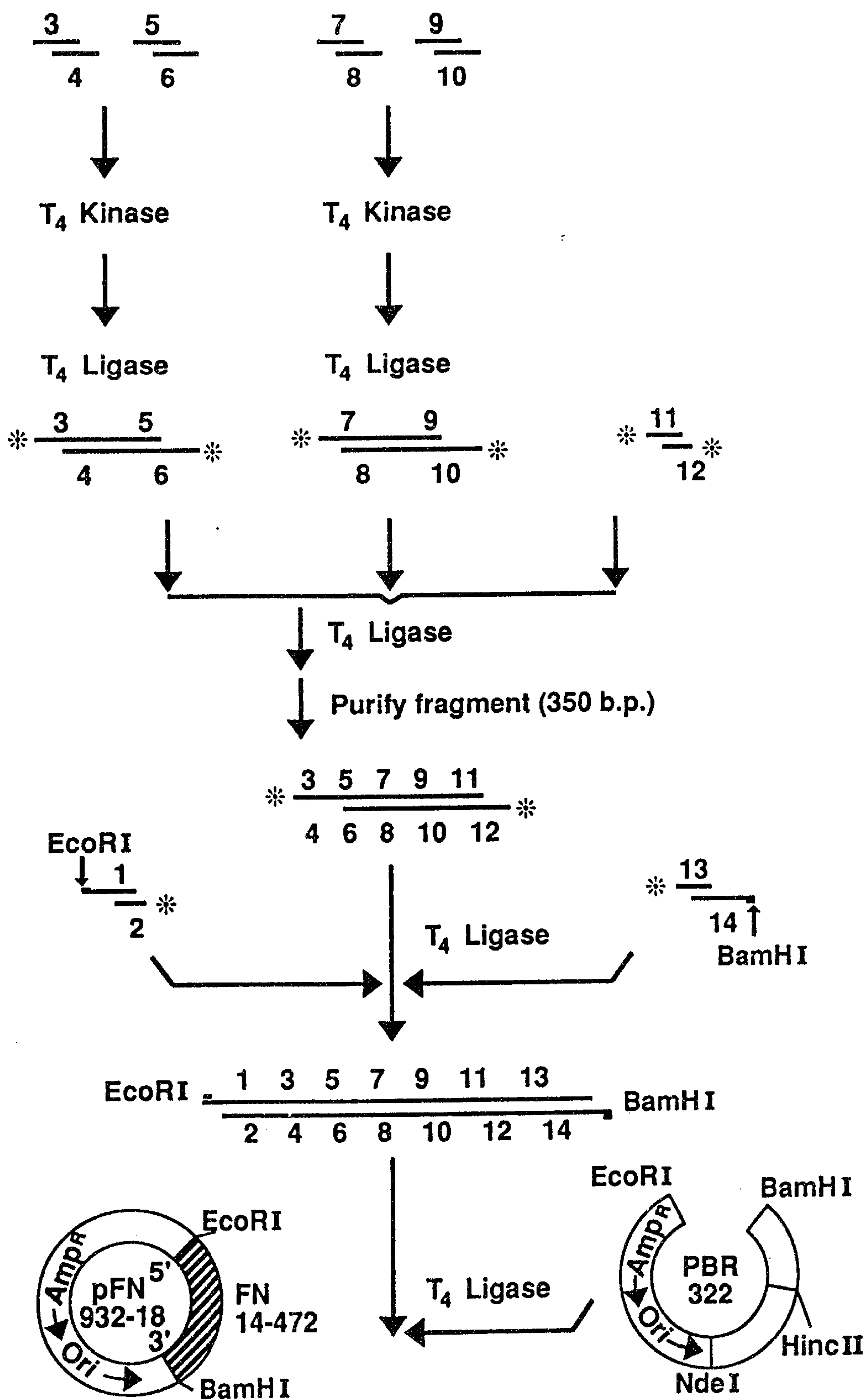
Figure 42B

Pair 5
9 5'- GACTCCATGATCTGGGACTGTACCTGCGGGGCTGGGCGAGGGAGAAATAAGCTGTACC - 3'
#10 GATTCTGAGGTACTAGACCCCTGACATGGACGTAGCCCCCGACCCGCTCCCTCTTATTC - 5'

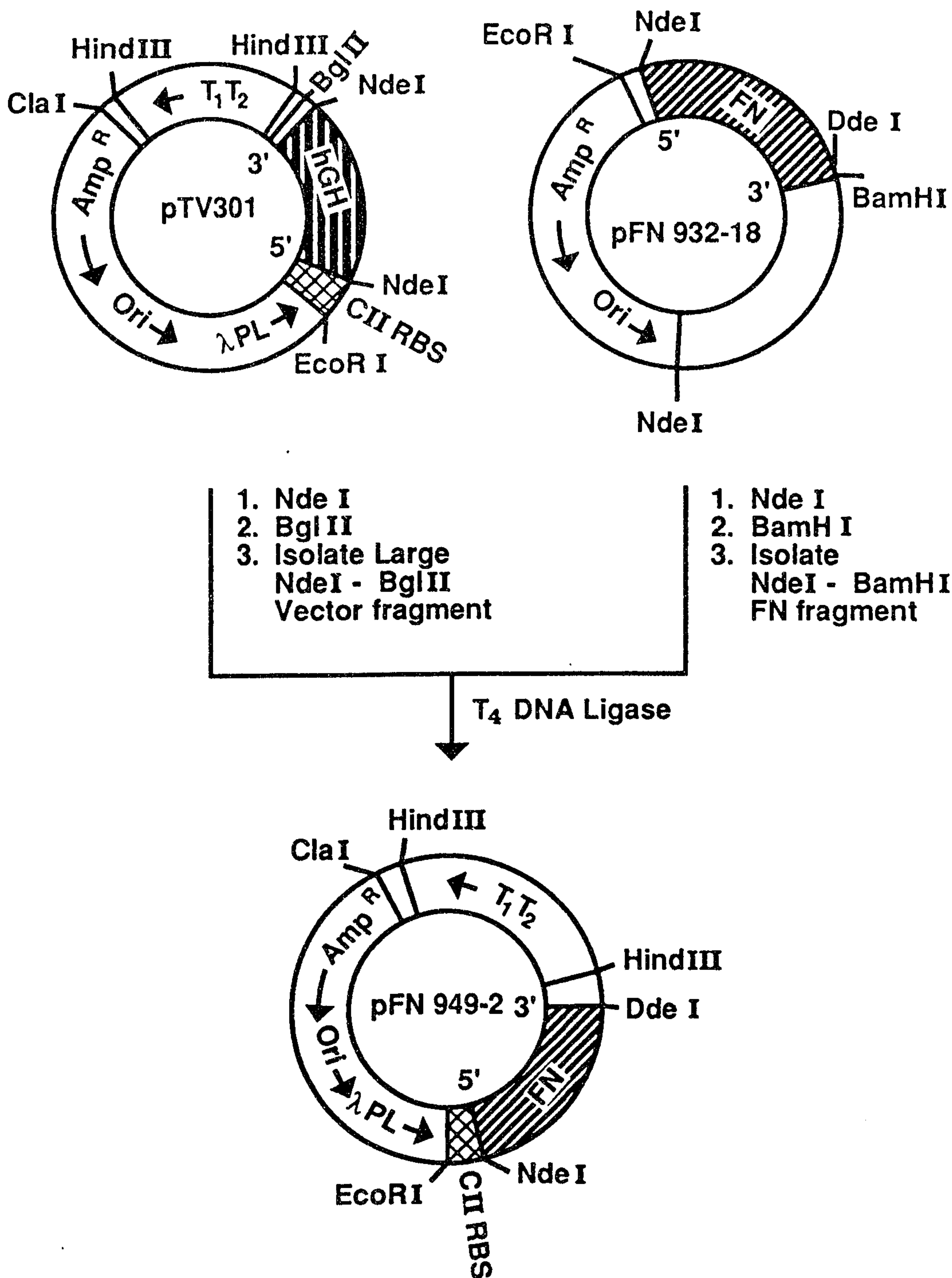
Pair 6
#11 5'- ATCGCCAAACGCTGCCATGAAGGGGTCACTCCTACAGATTGGTGACACCTGGAGGAGACCACATGAGACT - 3'
#12 GACATGGTAGCGTTTGGACGGTACTTCCCCAGTCAGGATGGTCTAACCACCTGTGGACCTCCTCTGGTGTACTCTGACCAACCAA - 5'

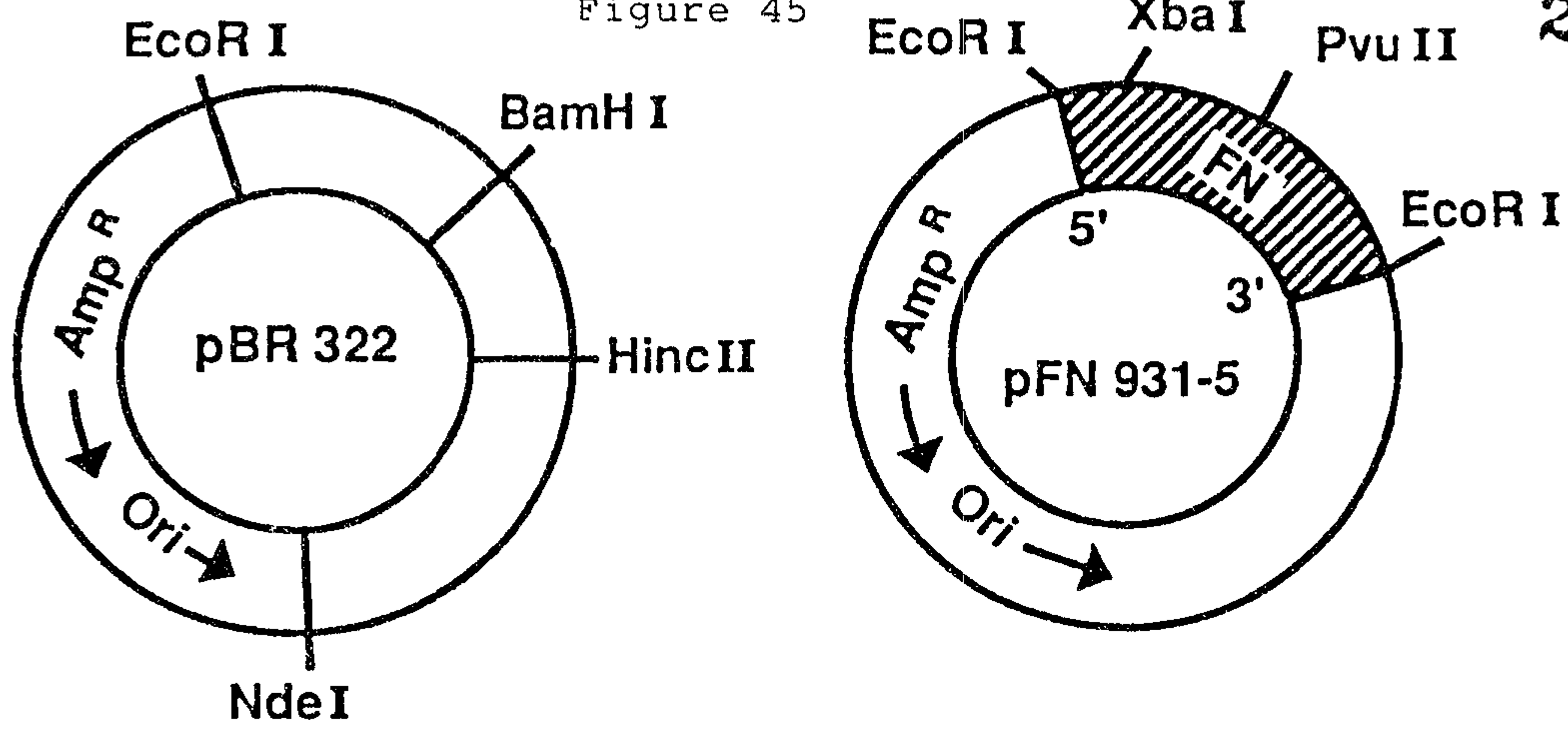
Pair 7
#13 5'- GGTGGTTACATGTTAGAGTGTGTGTCTTGGTAATGGAAAAGGAGAAATGGACCTGCCAAGCCCCATAGCTGAG - 3'
#14 3'- TGTACAATCTCACACACACAGAACCATTTACCTTTTCCCTCTTACCTGGACGTTTCGGGTATCGGACTCCTAG - 5'

Synthesis of F.N. Gene (14 - 472 b.p.)



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Figure 44





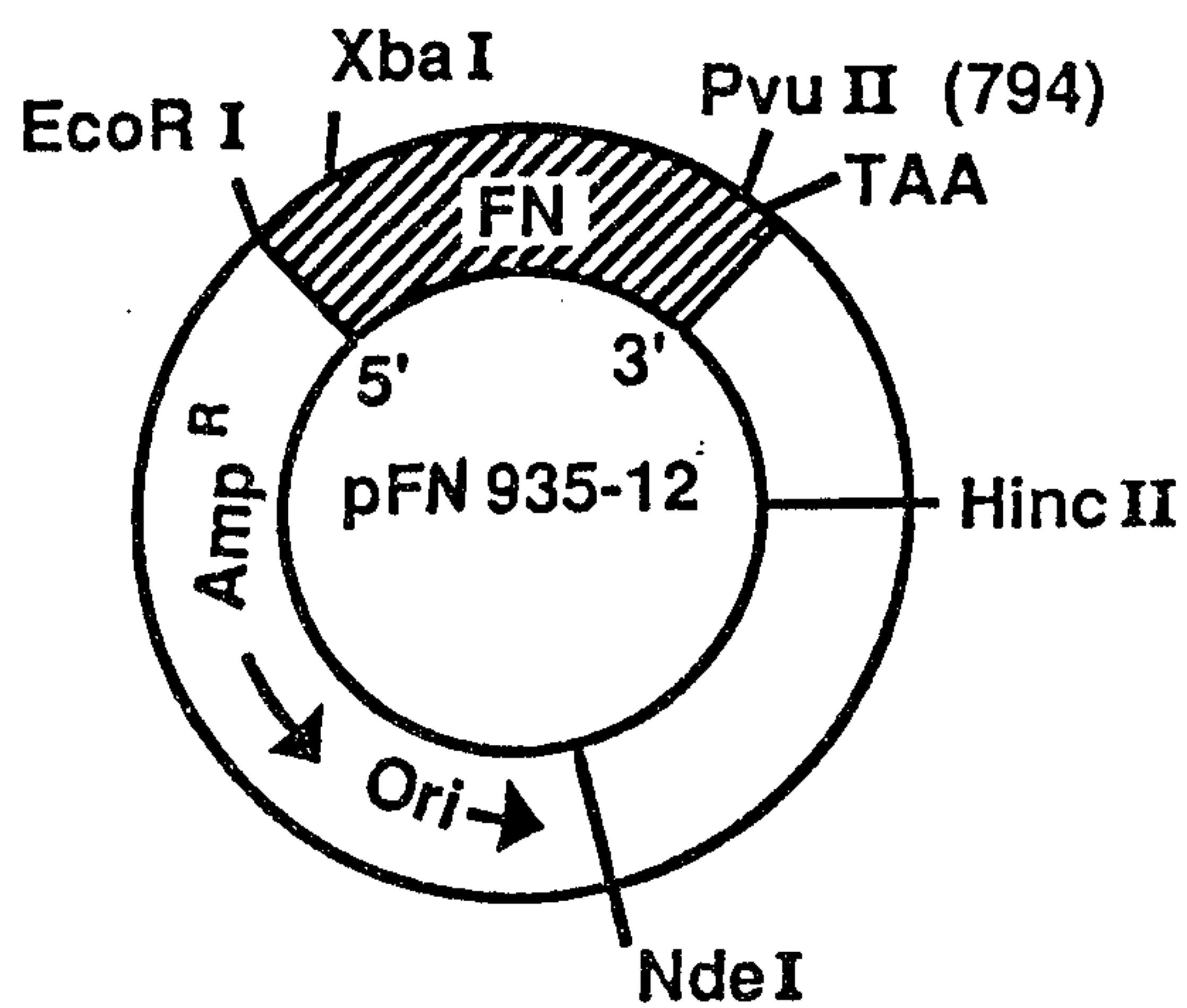
1. EcoRI
2. BamHI
3. Isolate Large EcoRI - BamHI fragment

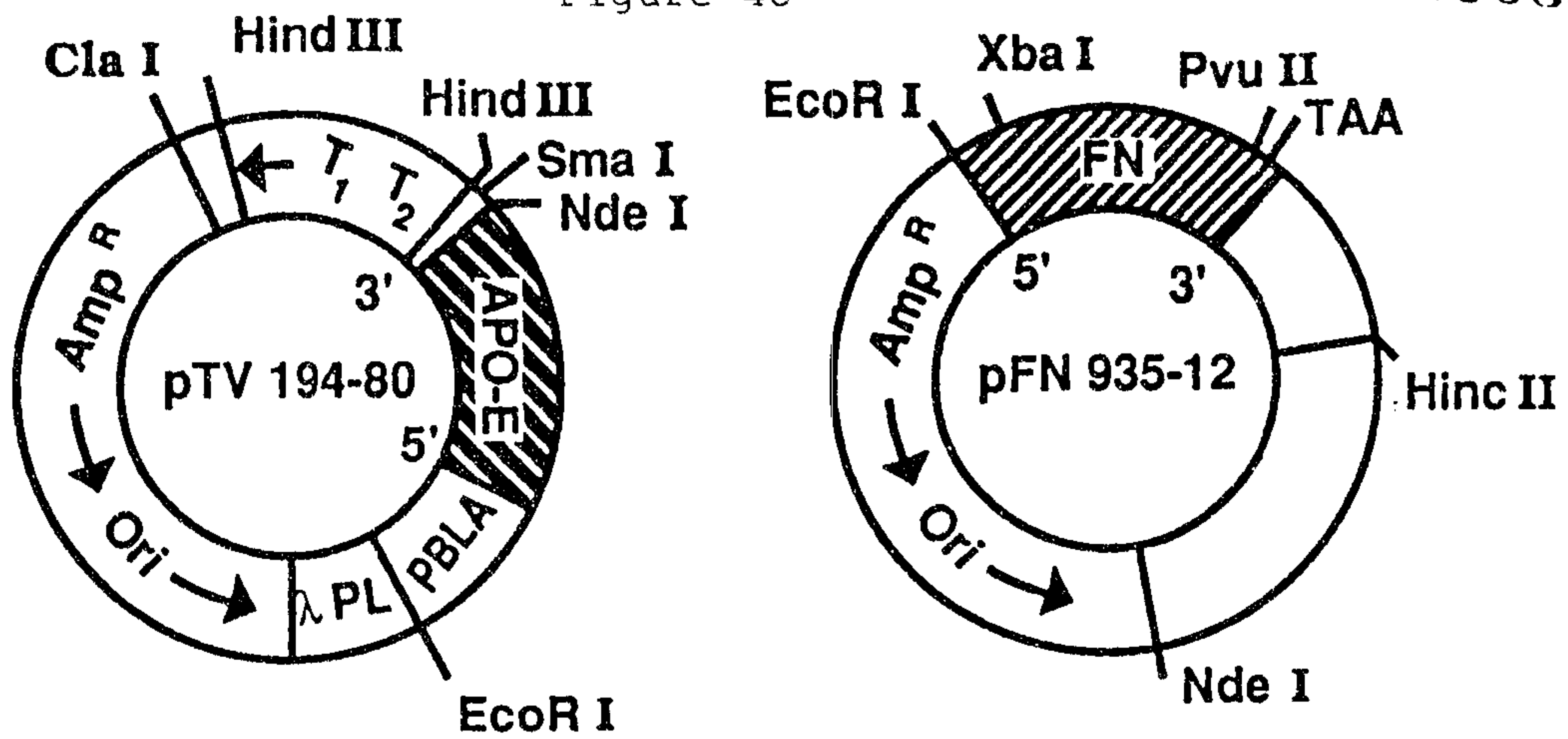
1. EcoRI
2. Pvu II
3. Isolate Large EcoRI - PvuII FN fragment

Synthetic Linker
5' -CTGTTTAAGCA-3'
3' -GACAAATTCGTCTAG-5'

Kinase

T₄ DNA Ligase





1. EcoRI
2. Sma I
3. Isolate large
EcoRI - SmaI fragment

1. EcoRI
2. Hinc II
3. Isolate
EcoRI - Hinc II
FN fragment

T₄ DNA Ligase

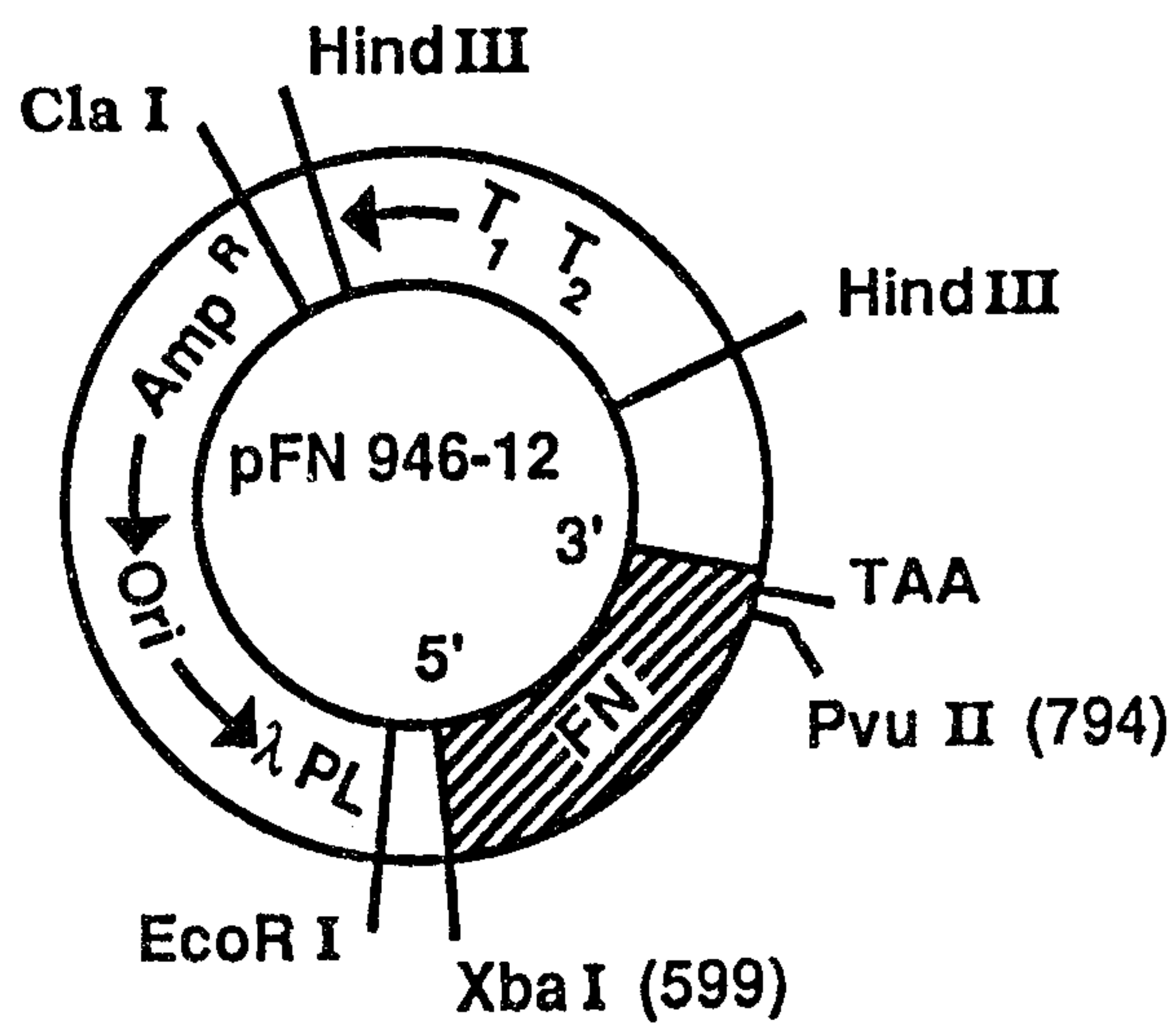


Figure 47

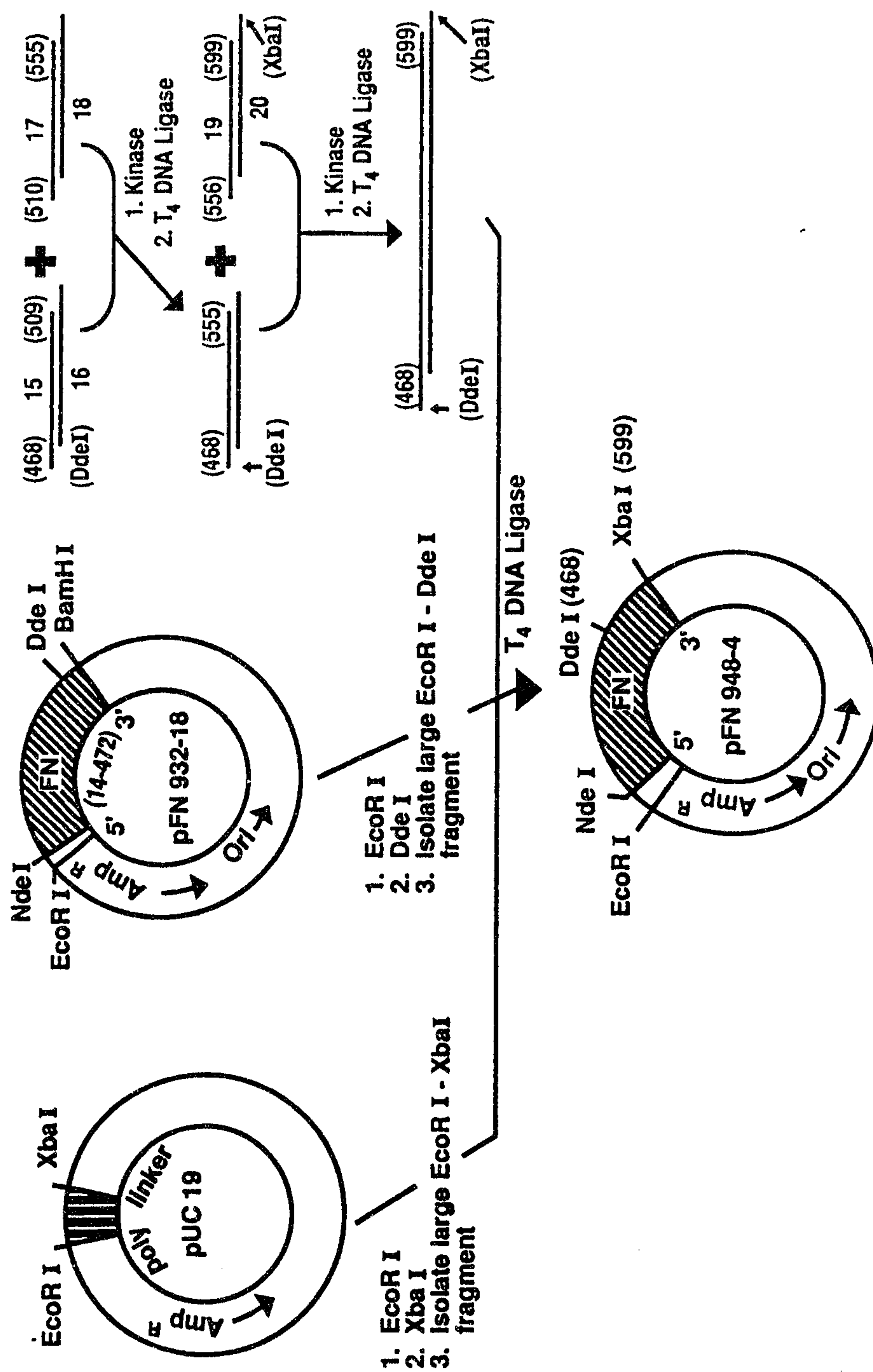


Figure 48

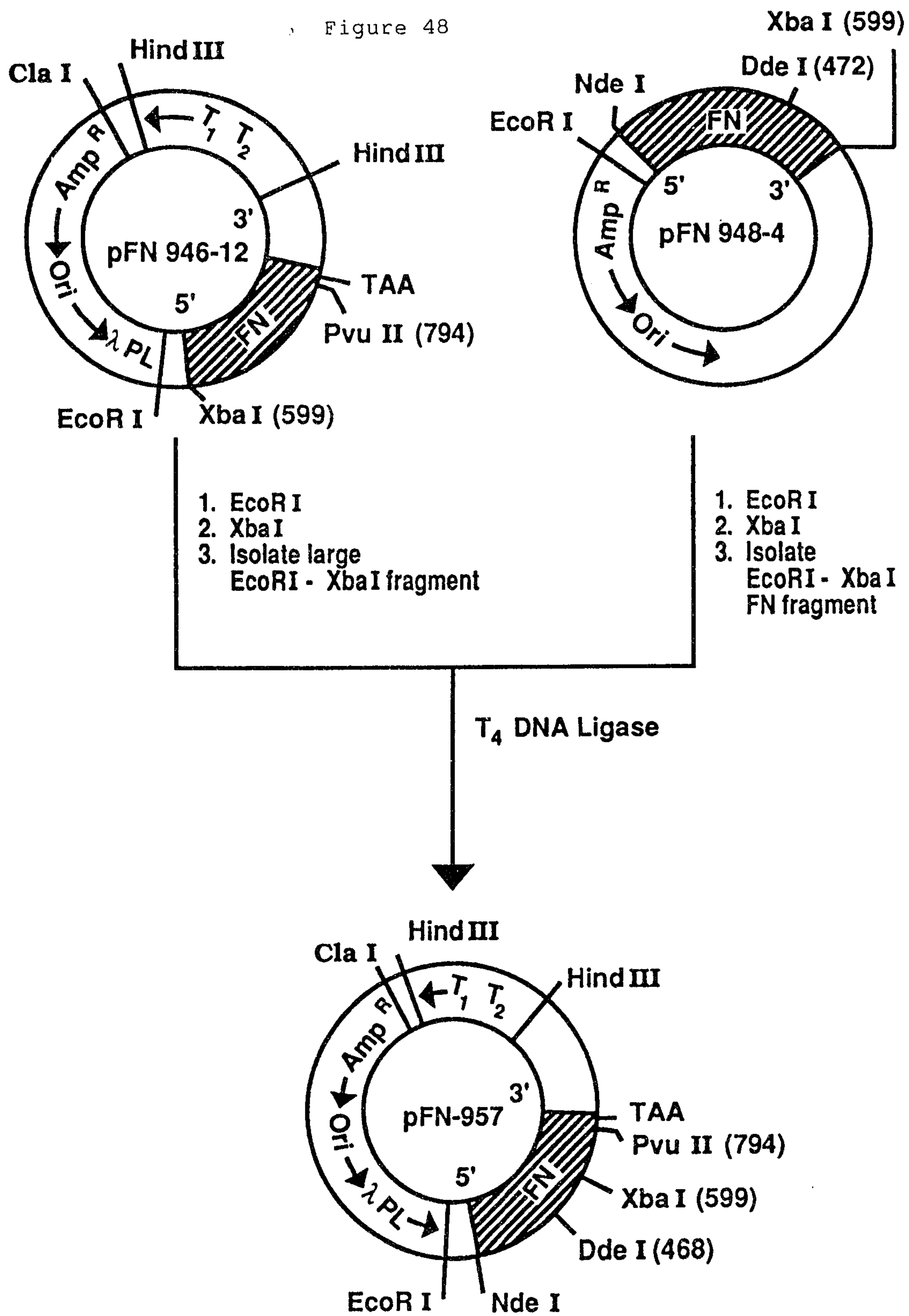
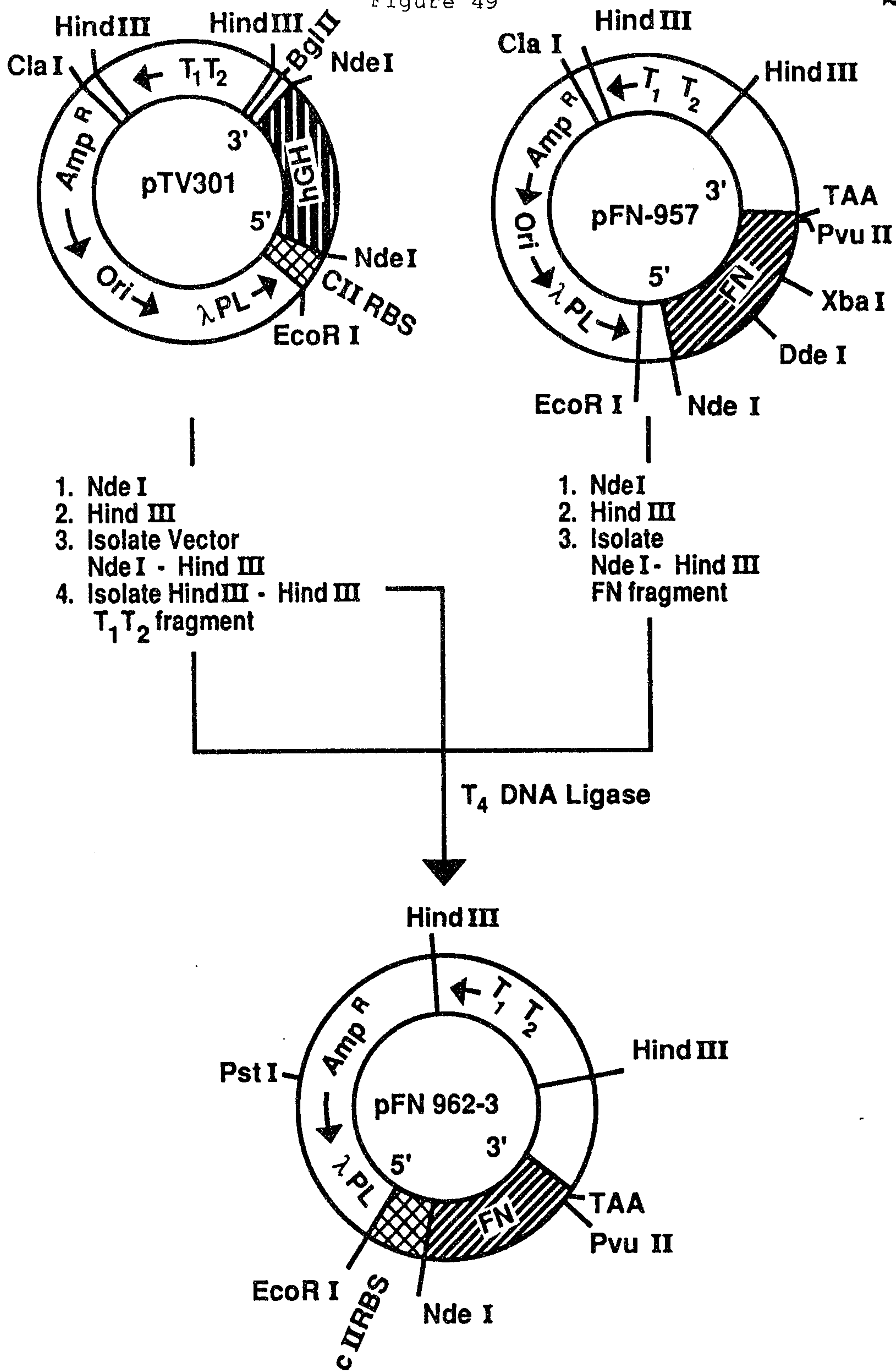
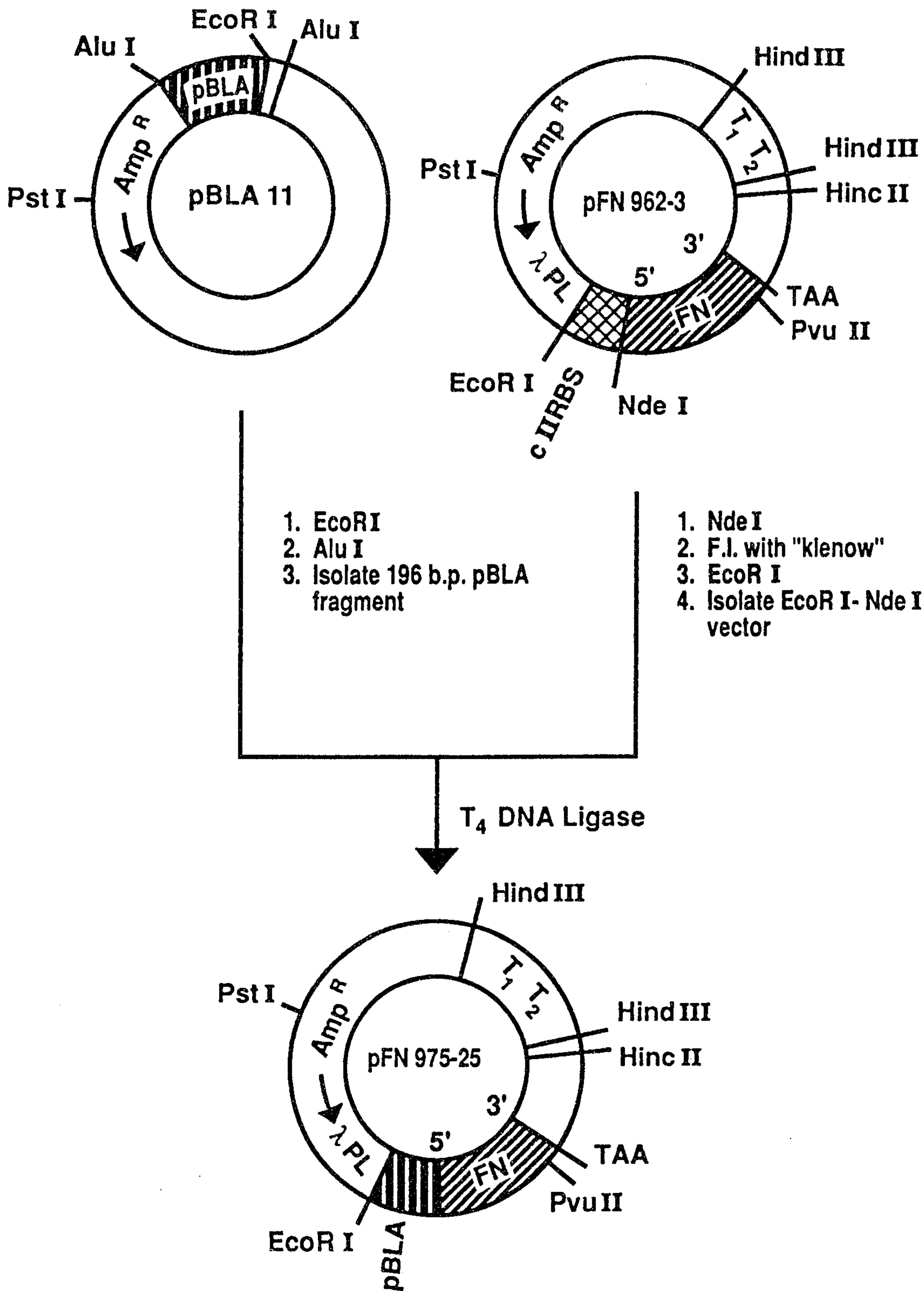


Figure 49

2006929

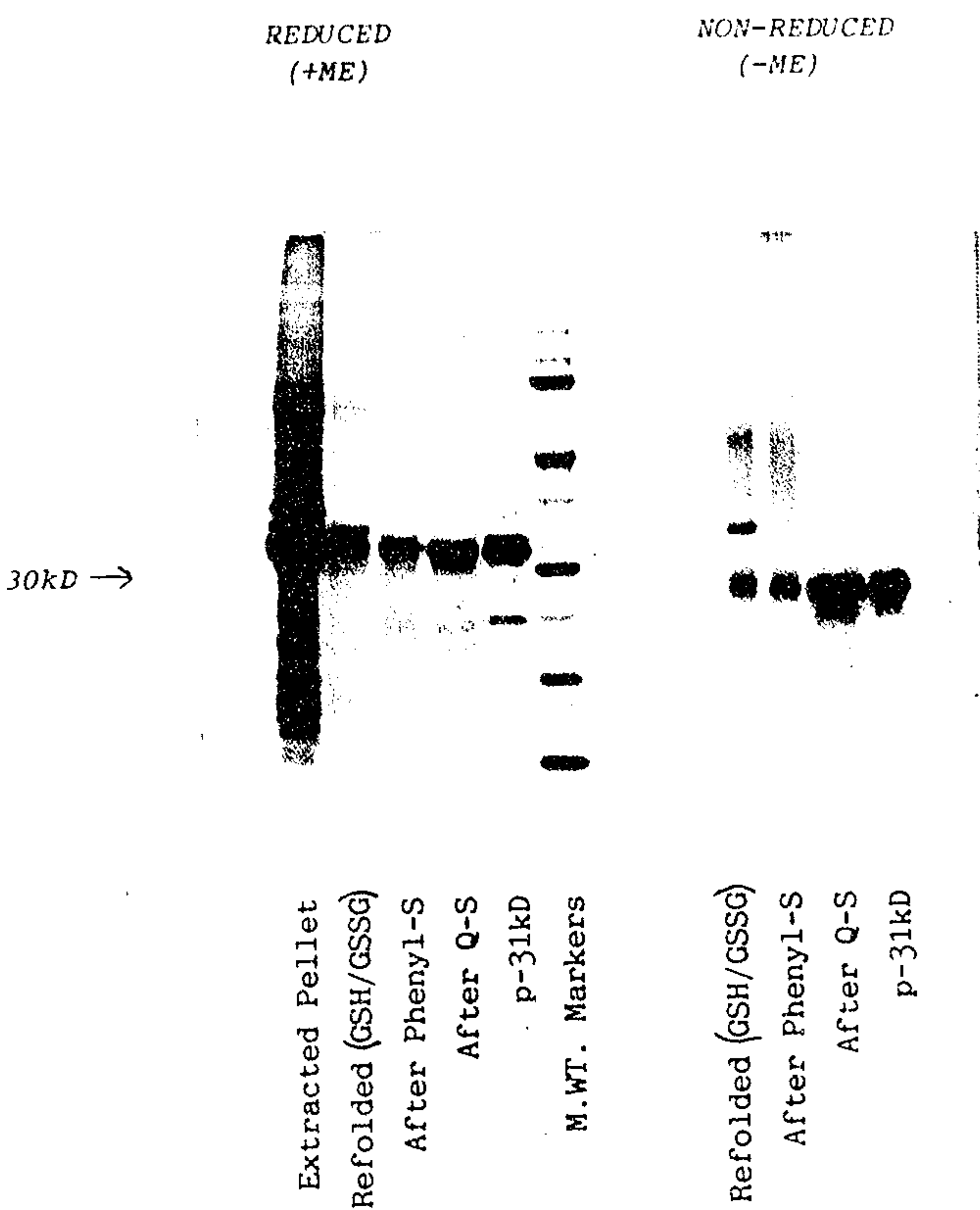


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Figure 50



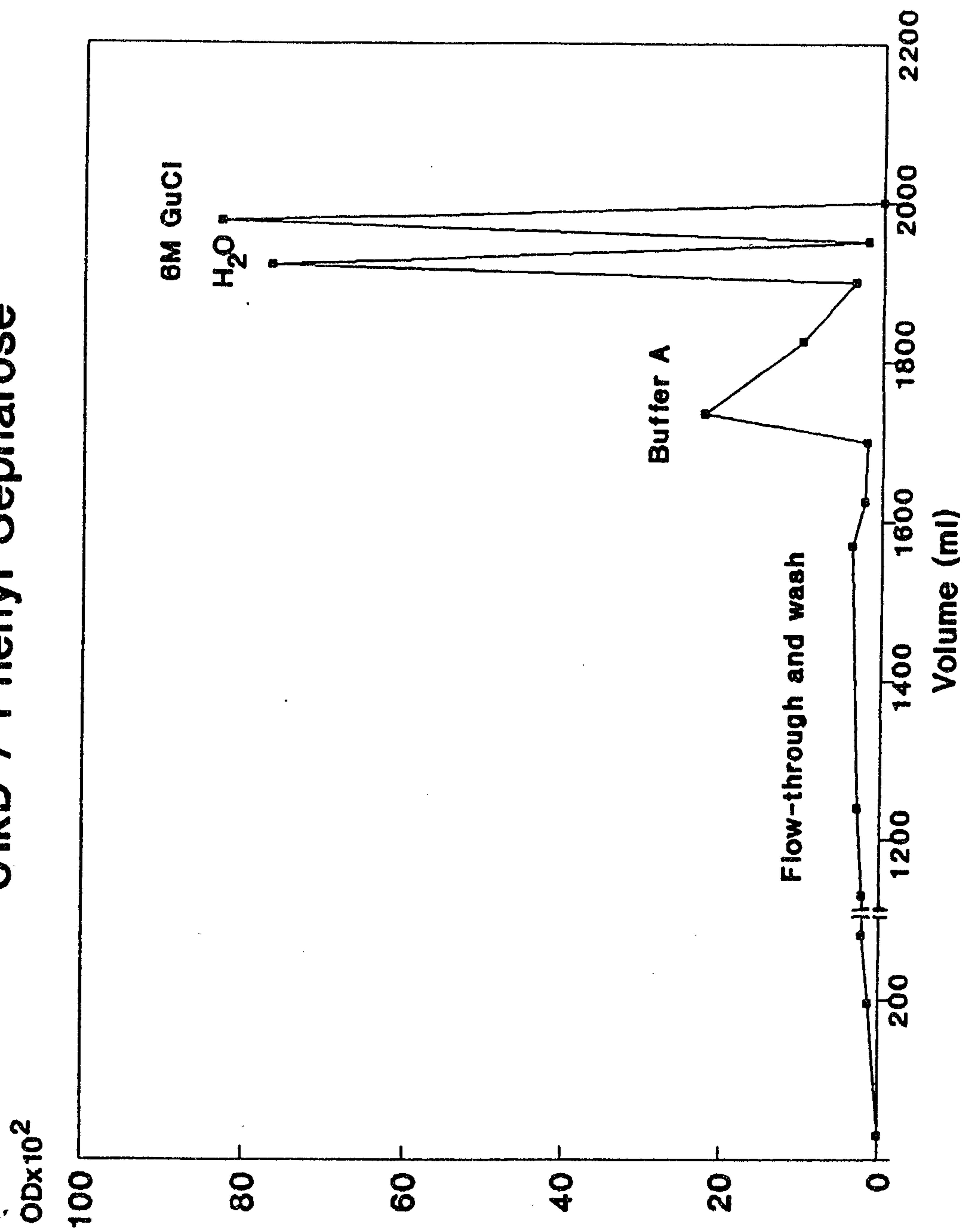
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Figure 51



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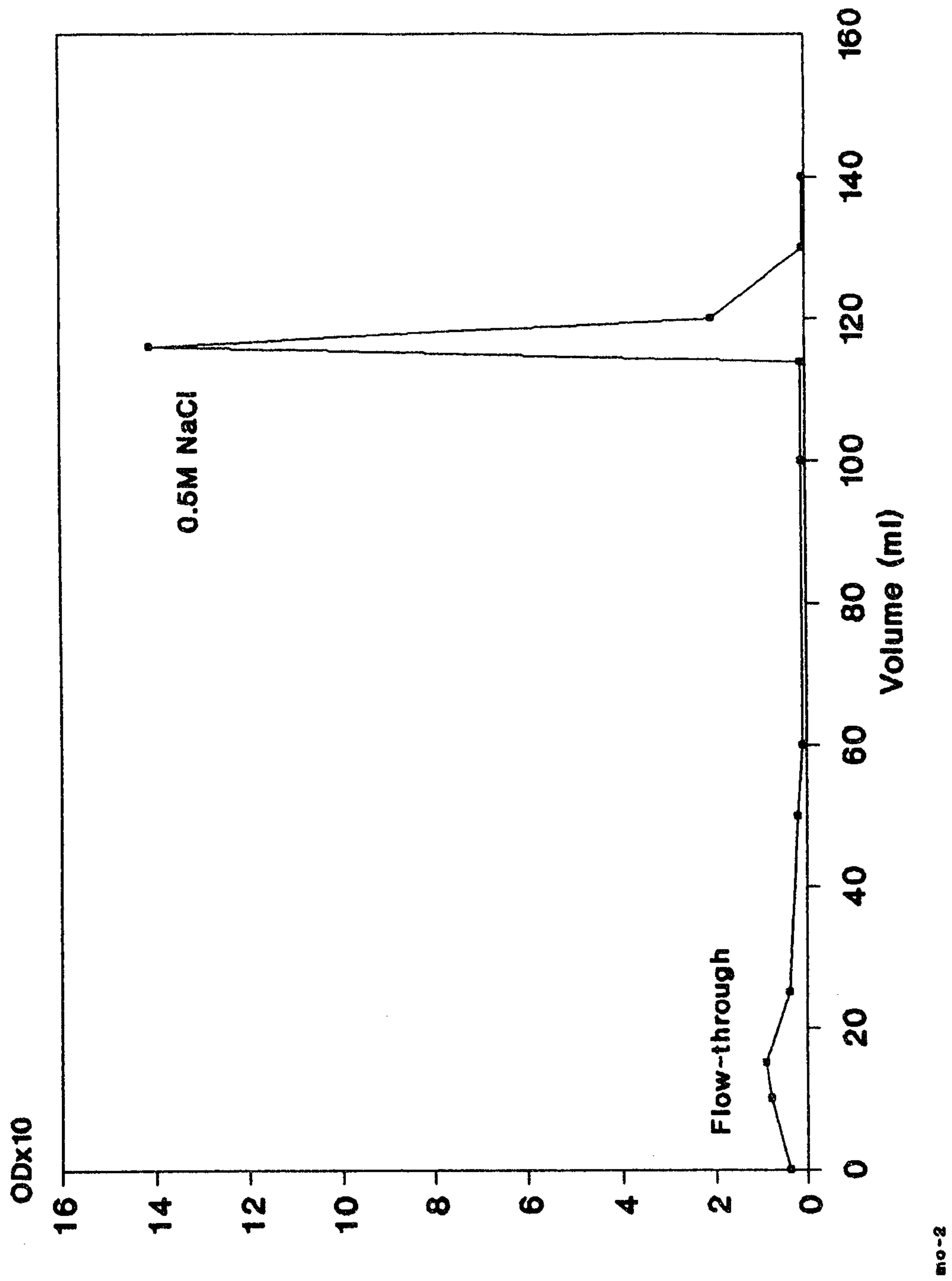
Figure 52
31kD / Phenyl-Sepharose



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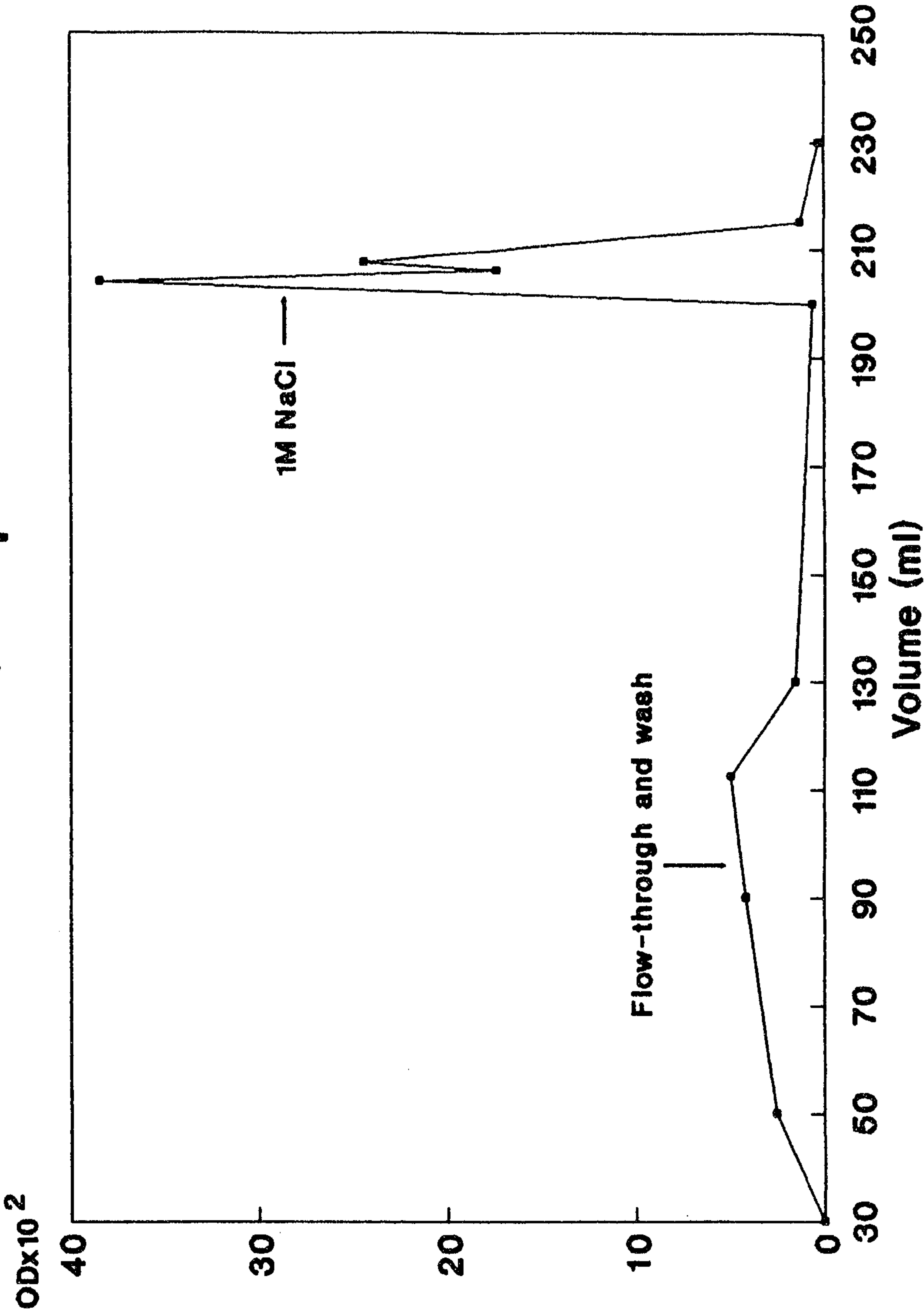
Figure 53
31kD/Heparin-Sepharose



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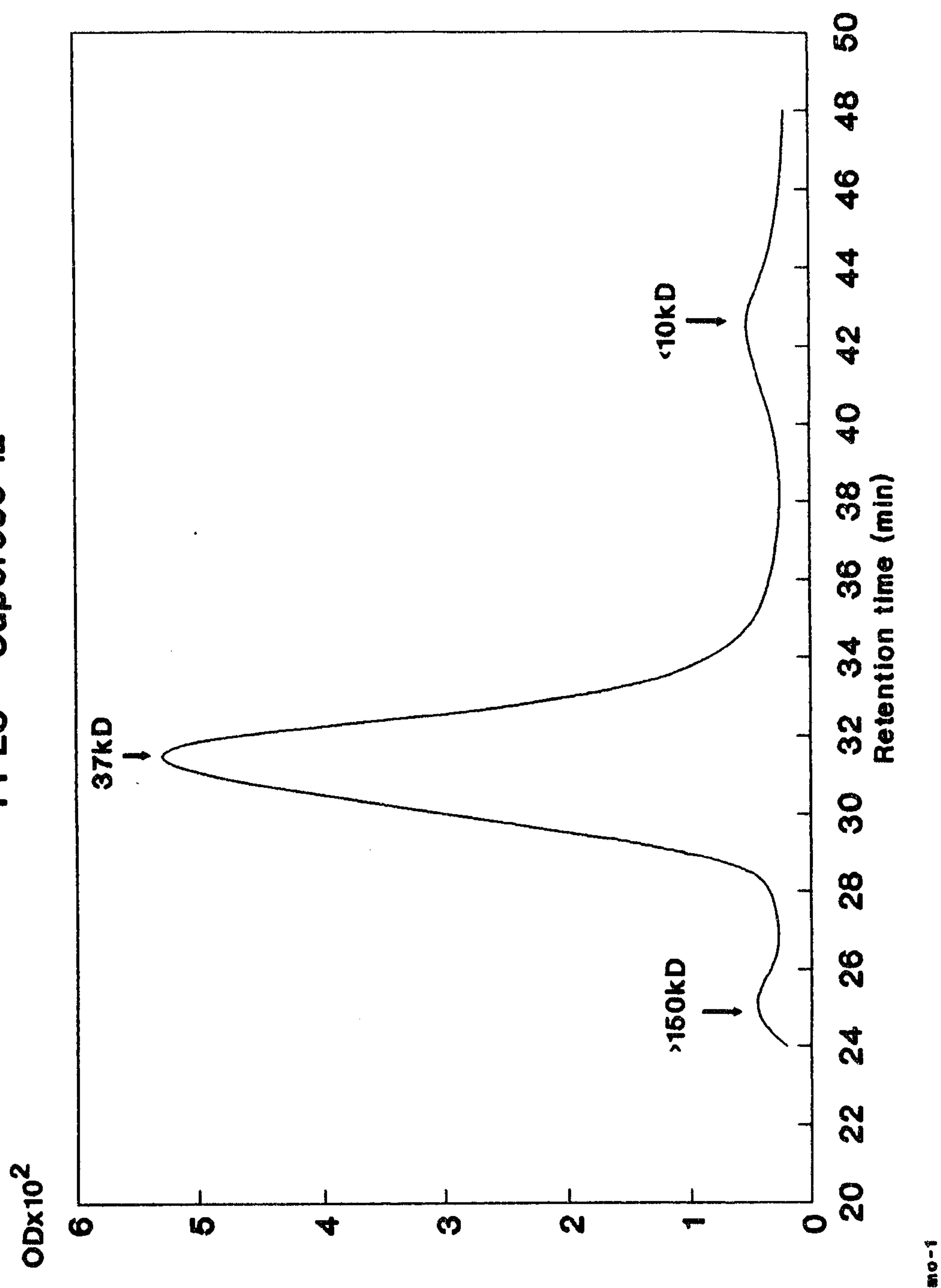
Figure 54

31kD / Q-Sepharose



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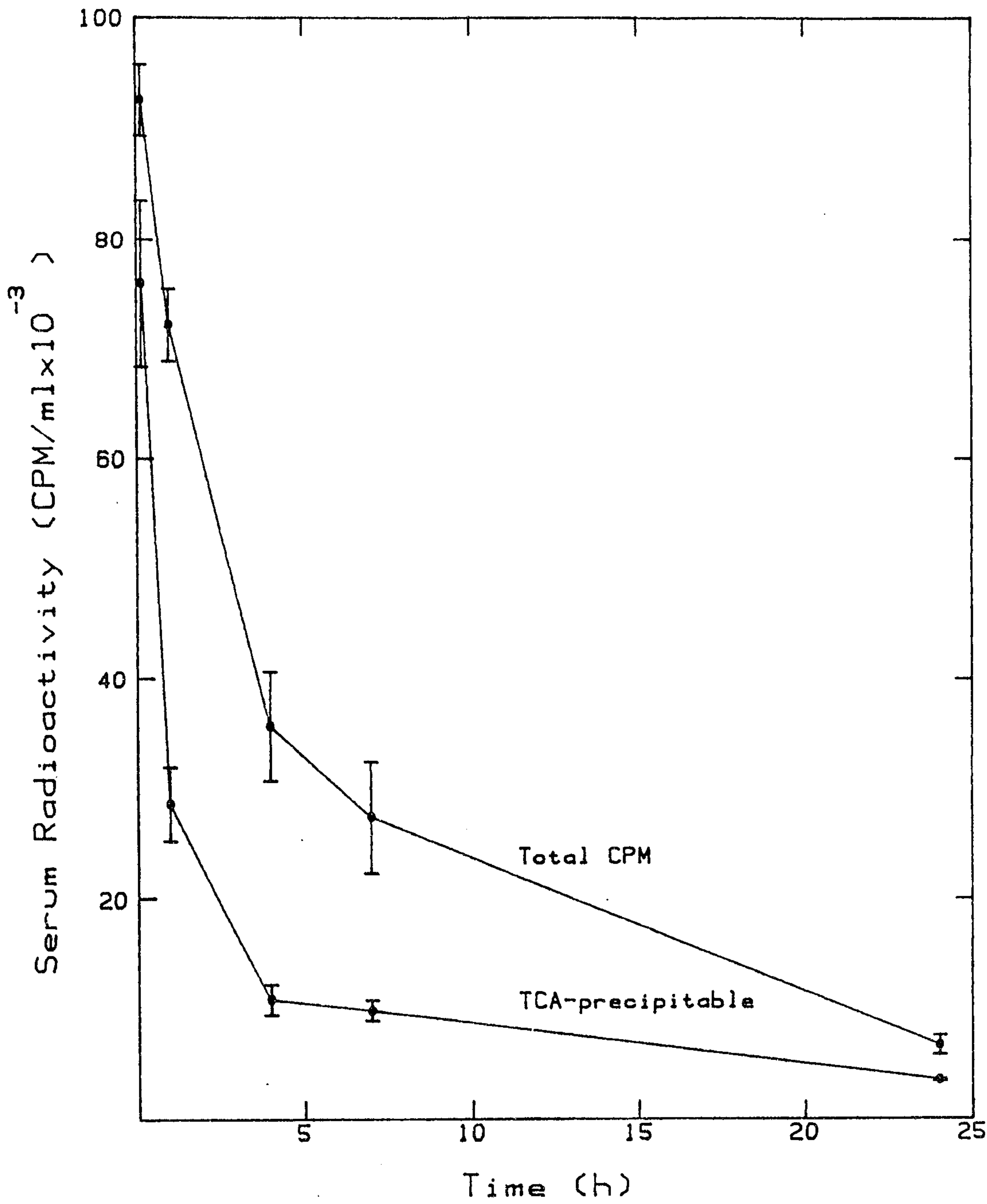
Figure 55
Mixture of rec. and plasmatic "31kD" FBD
FPLC - Superose 12



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Figure 56

Pharmacokinetics of Plasma-derived
31kD FBD Fragment of FN in Rats



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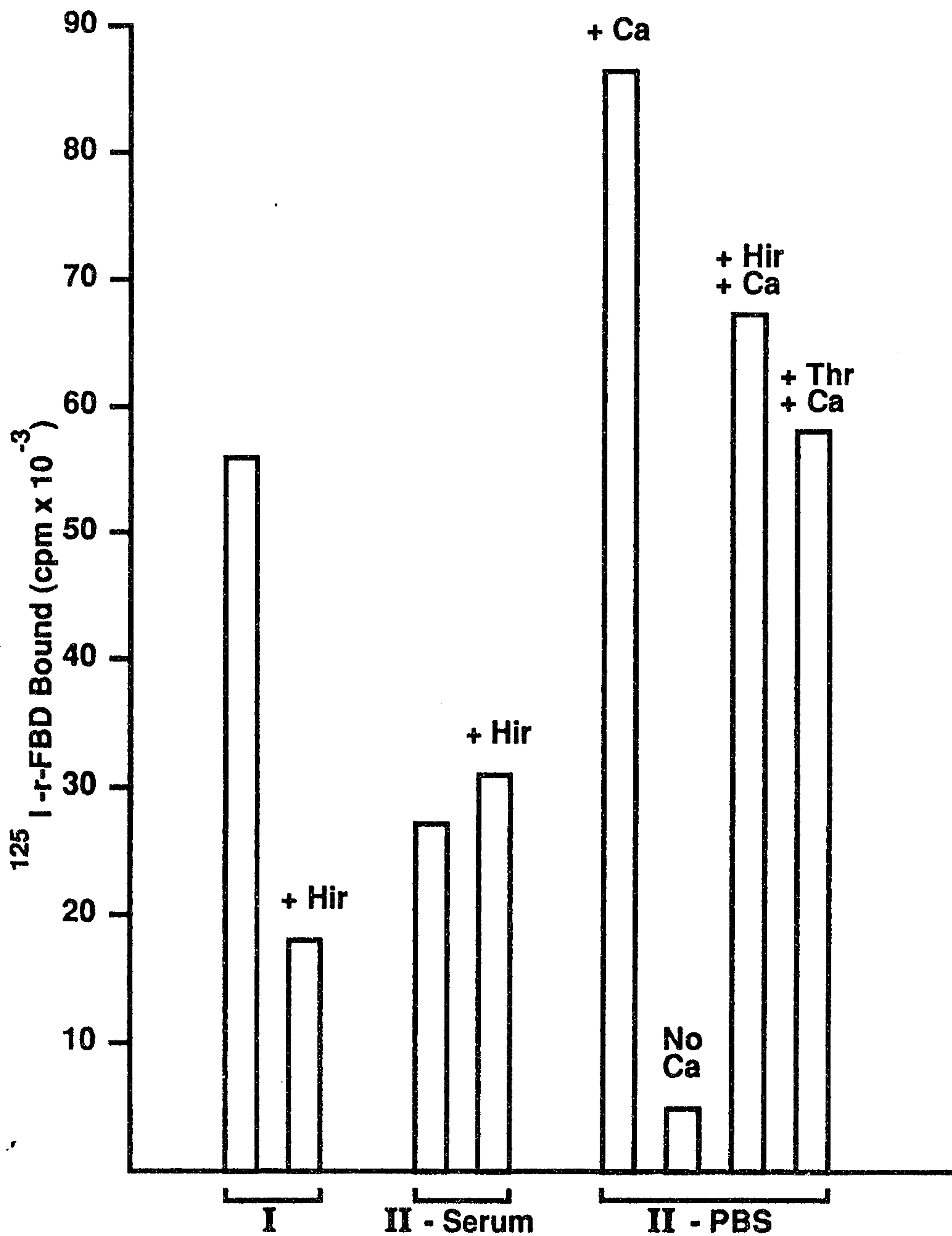
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Figure 57

Binding of ^{125} I-FBD to Fibrin;
Effect of Thrombin and Ca^{++} ions.

I = Binding while clot formation

II = Binding to preformed Fibrin clot

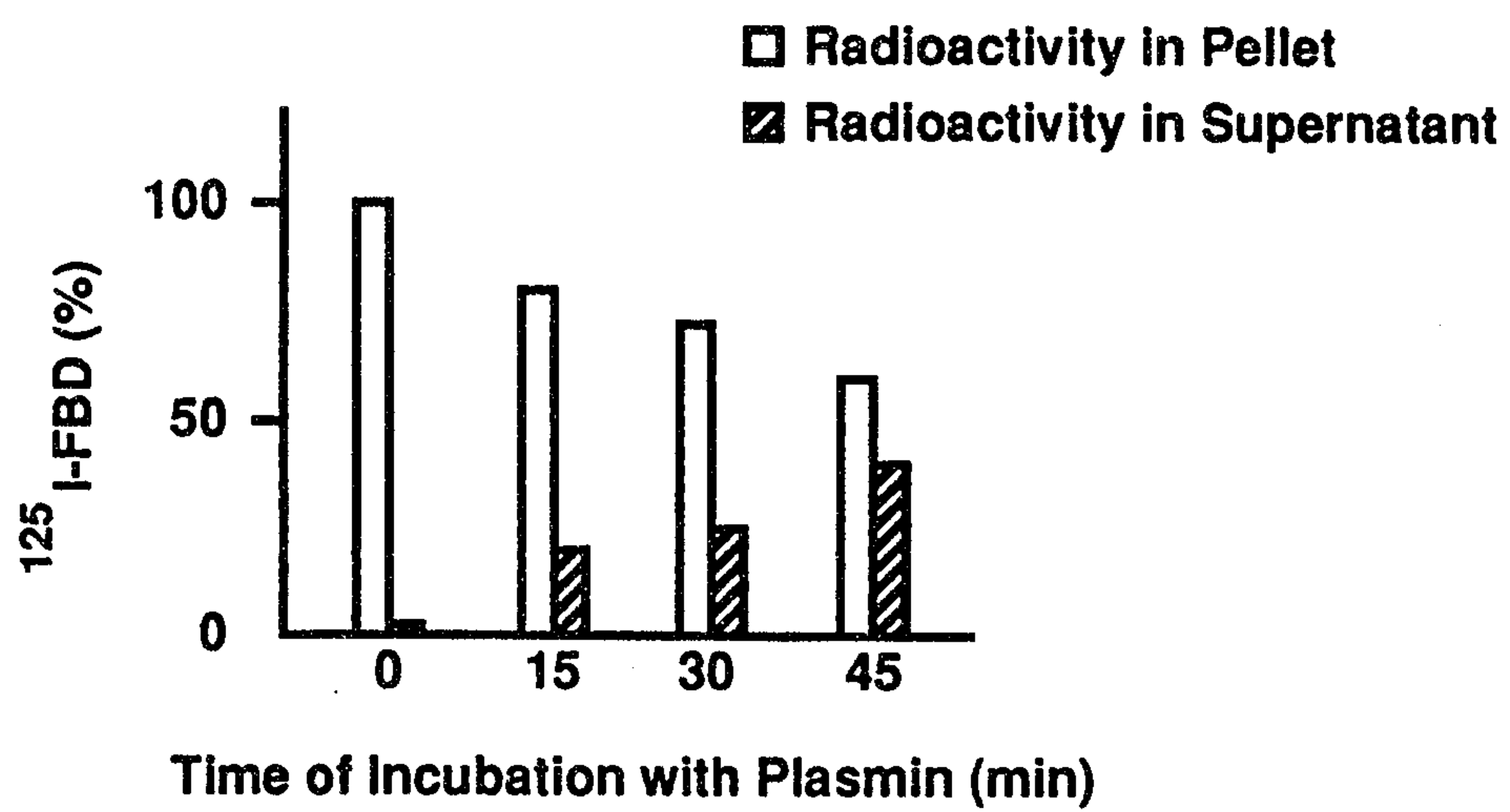


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Figure 58

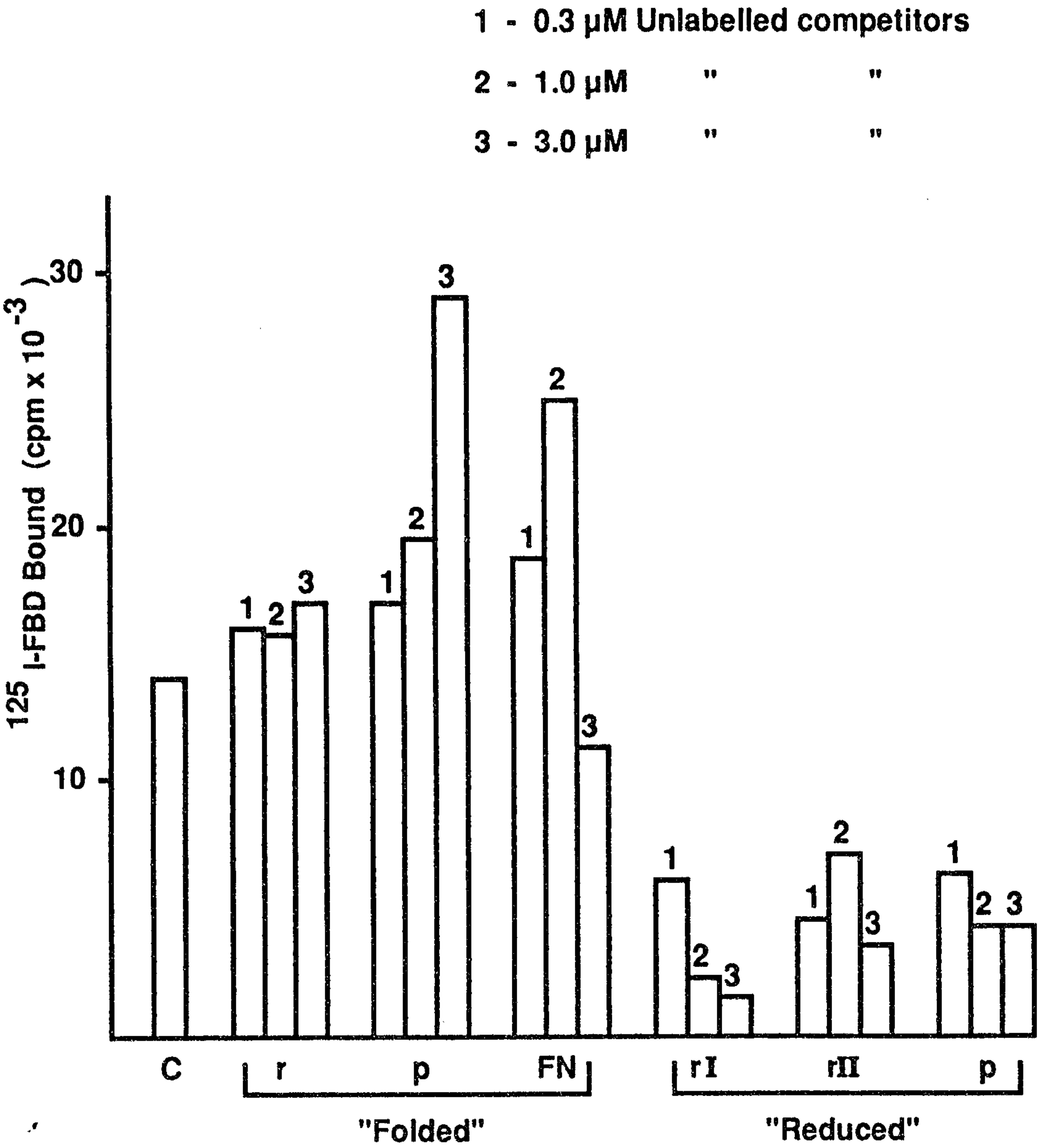
Release of ^{125} I-FBD from Fibrin Clot by Plasmin.

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Figure 59

Binding of ^{125}I -FBD during clot formation (Reaction I) ;
Effect of unlabelled FBD and related molecules.

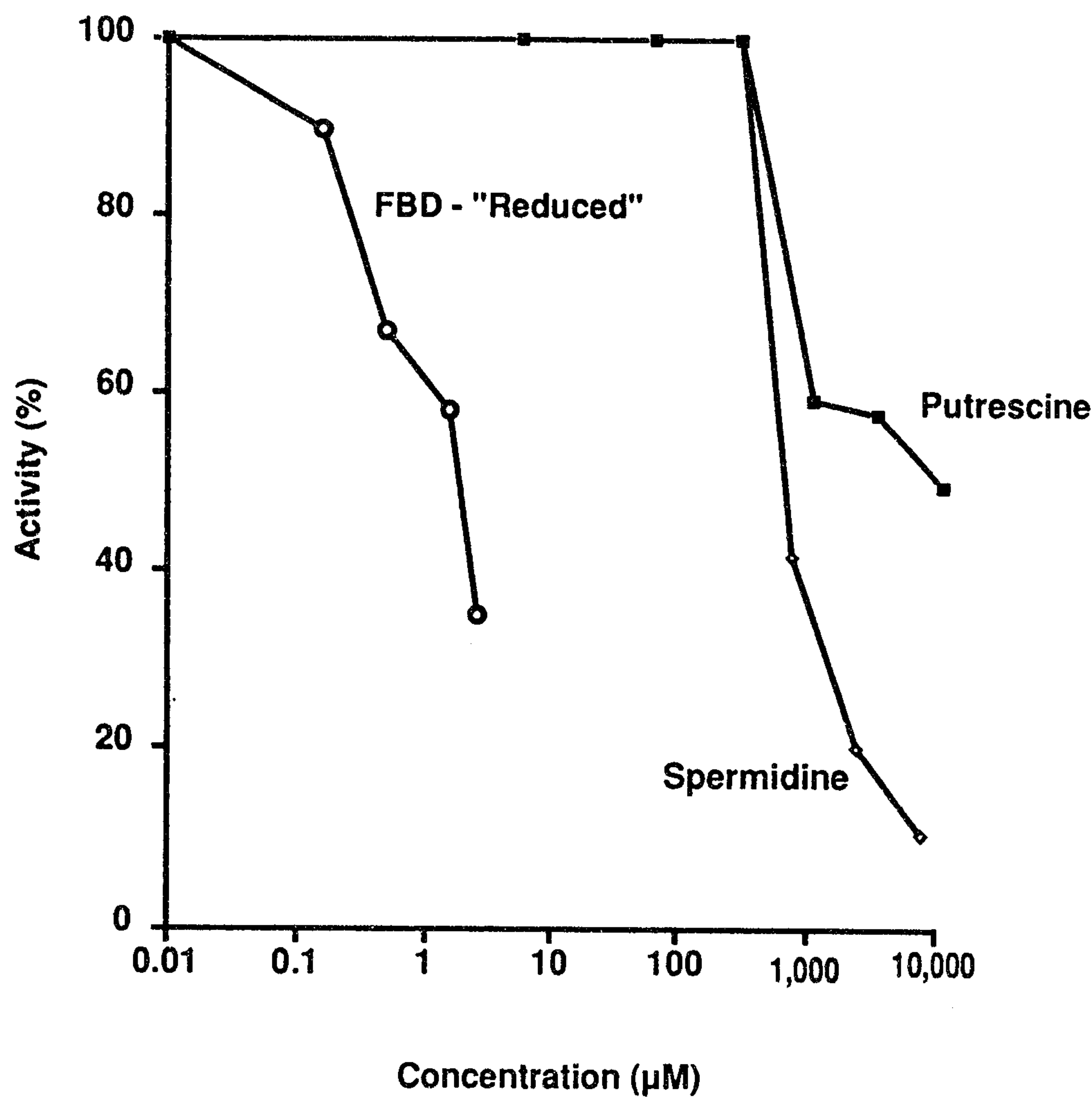


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Figure 60

Binding of ^{125} I-FBD to Fibrin (Reaction II);
Effect of Transglutaminase Inhibitors.

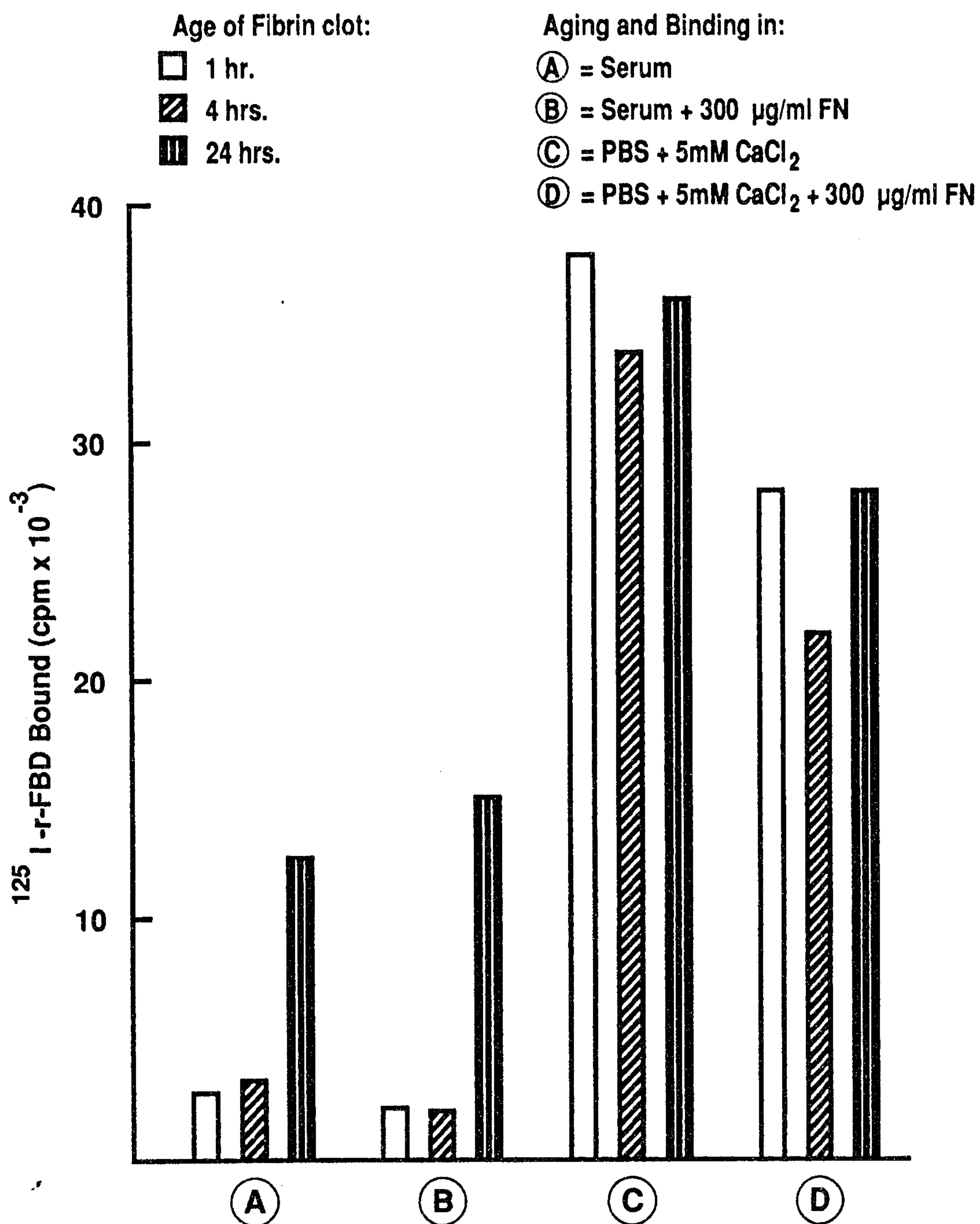


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Figure 61

Binding of ^{125}I -FBD to preformed Fibrin clot (Reaction II);
Effect of Fibrin clot Age on the Binding (aging at 37° C).

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Figure 62

Binding of FBD to "Naive" Thrombi

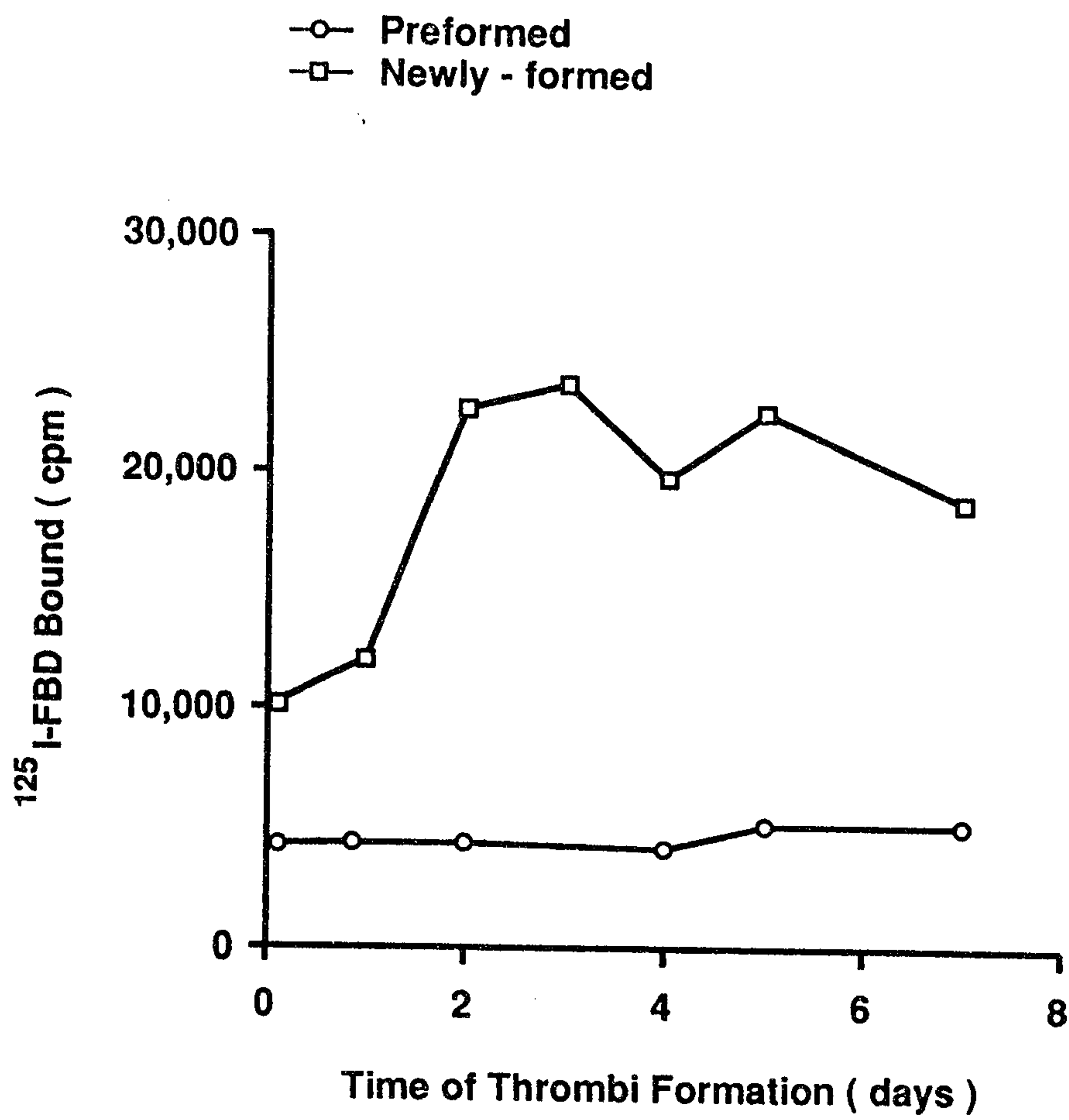
*Gowling, Strathy & Henderson*

Figure 63

Binding of FBD to Fibrin (Reaction I);
Effect of Transglutaminase ("TG")
and "Reduced" - FBD

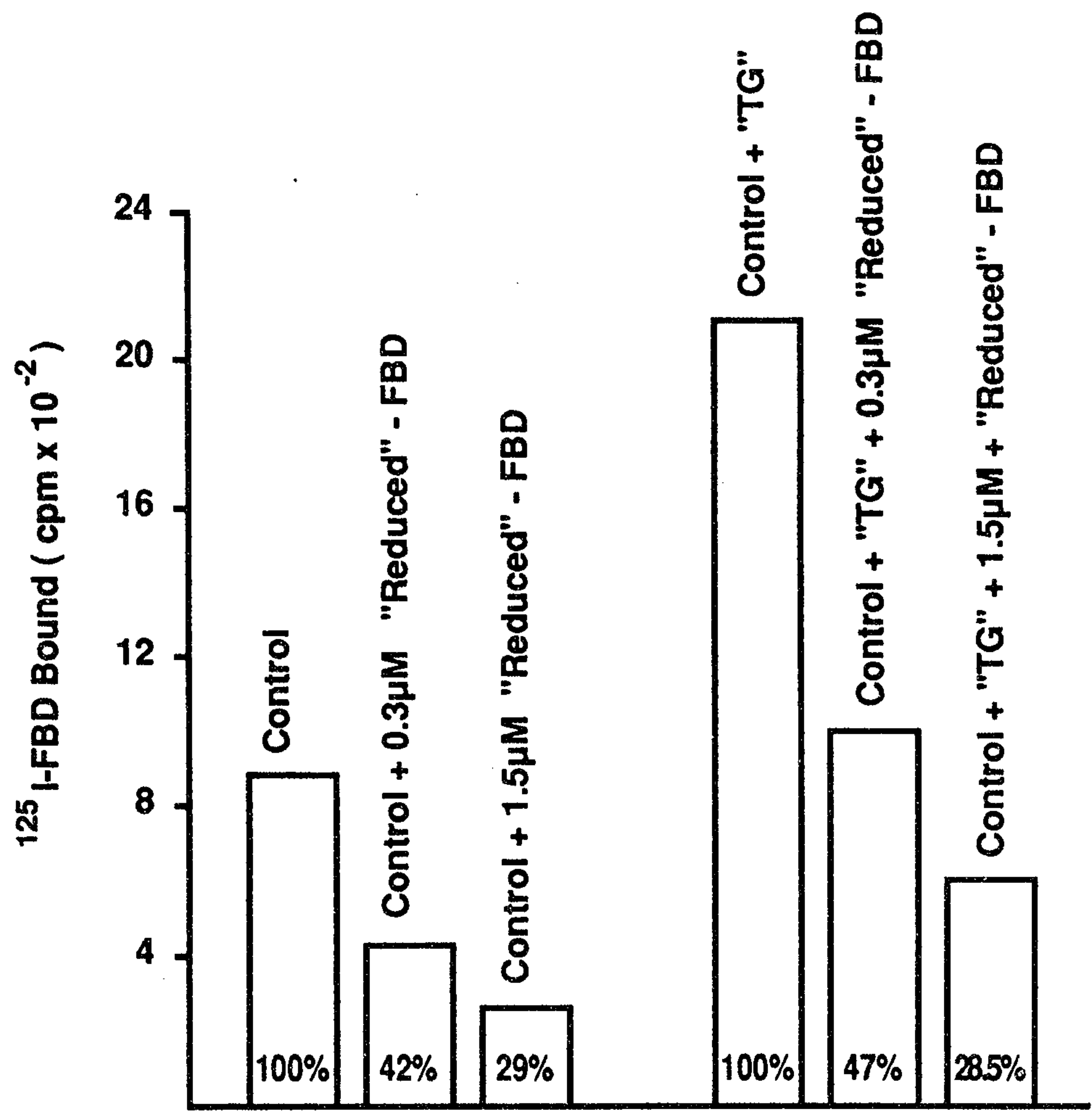
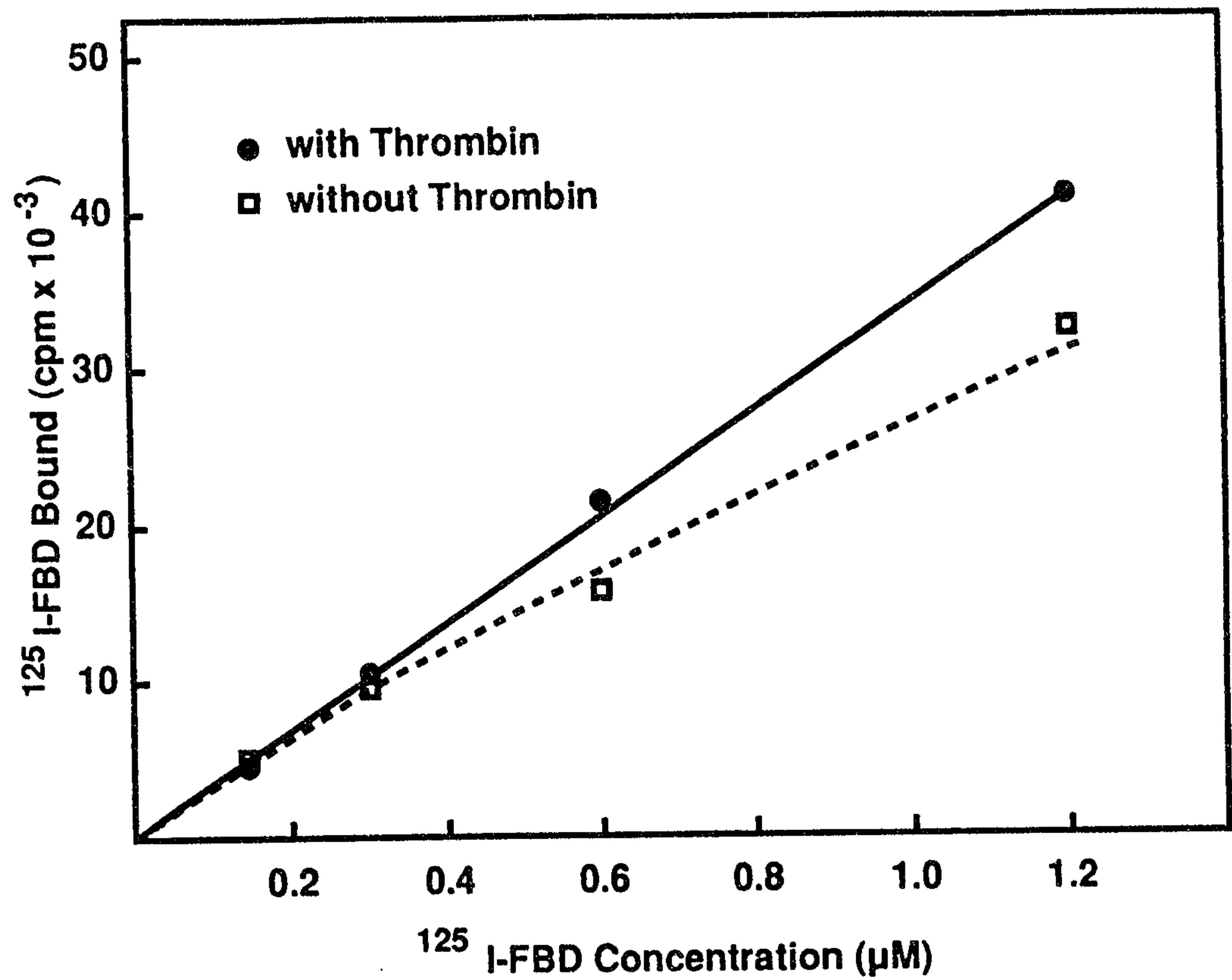


Figure 64

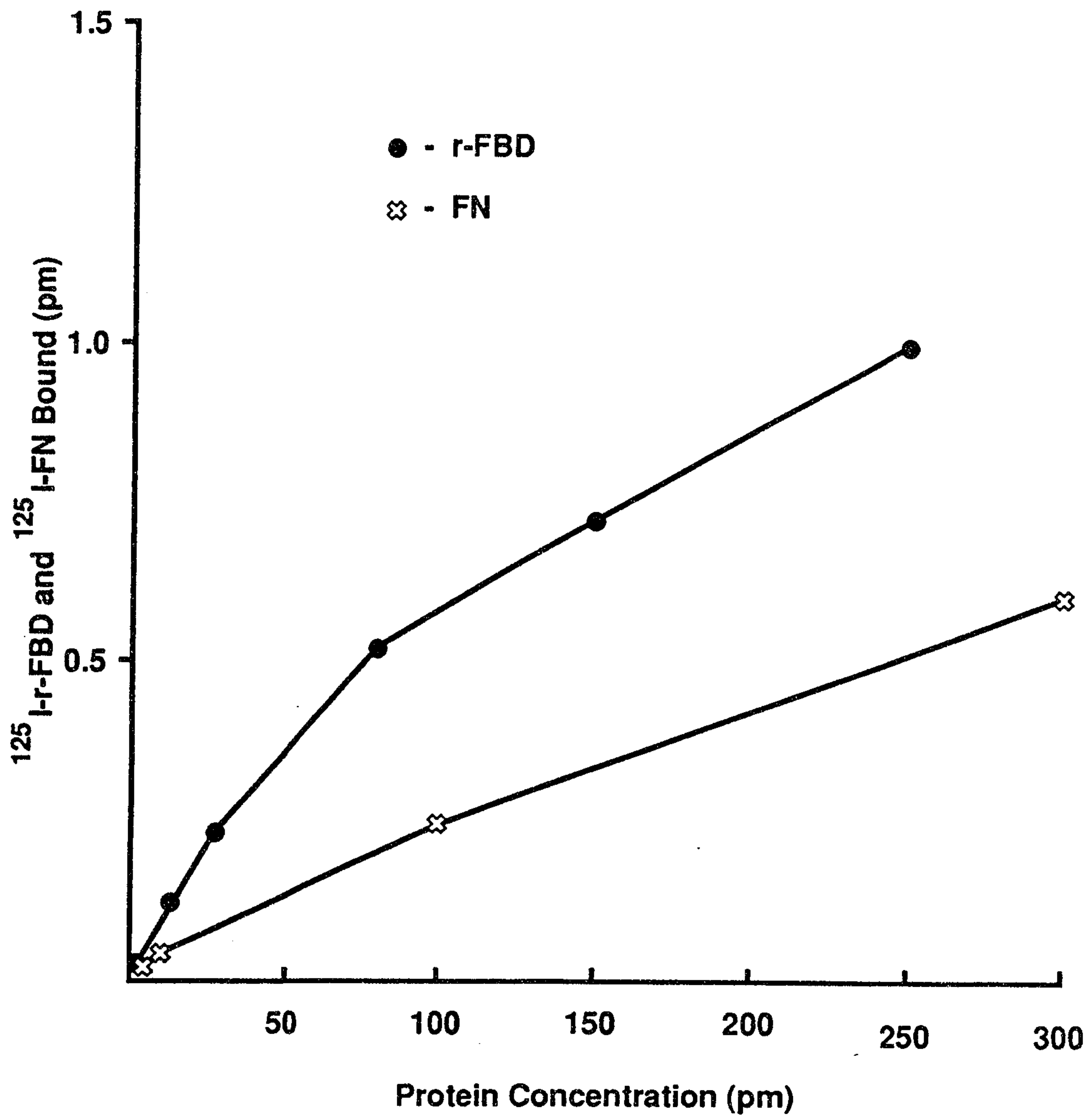
Binding of ^{125}I -FBD to ECM,
Effect of Ligand Concentration and Thrombin



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Figure 65

Binding of ^{125}I - (FN, r-FBD) to *S. aureus*

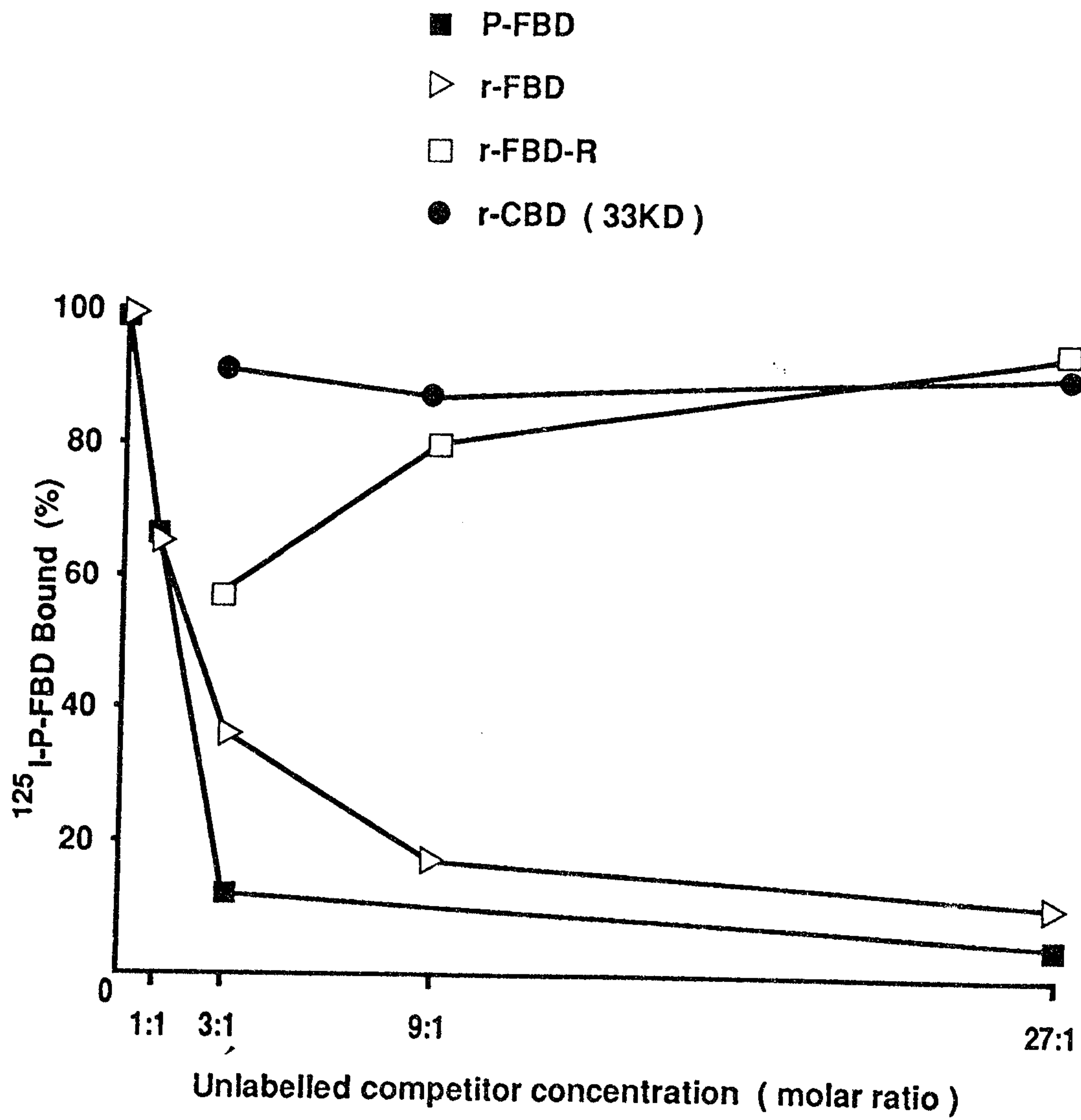


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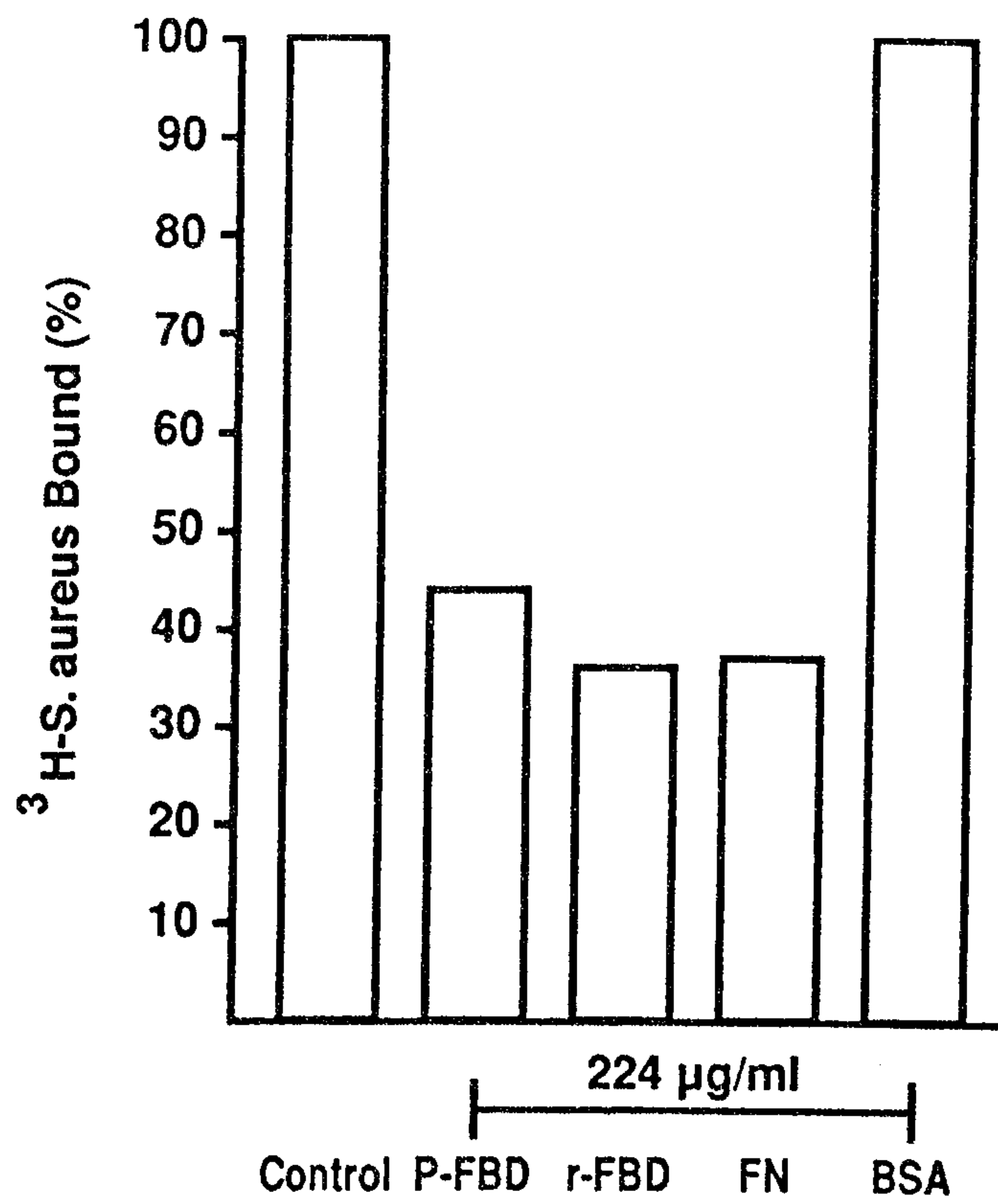
Figure 66

Binding of ^{125}I -FBD to *S. aureus*;
Competition with "Folded" and "Reduced" forms.

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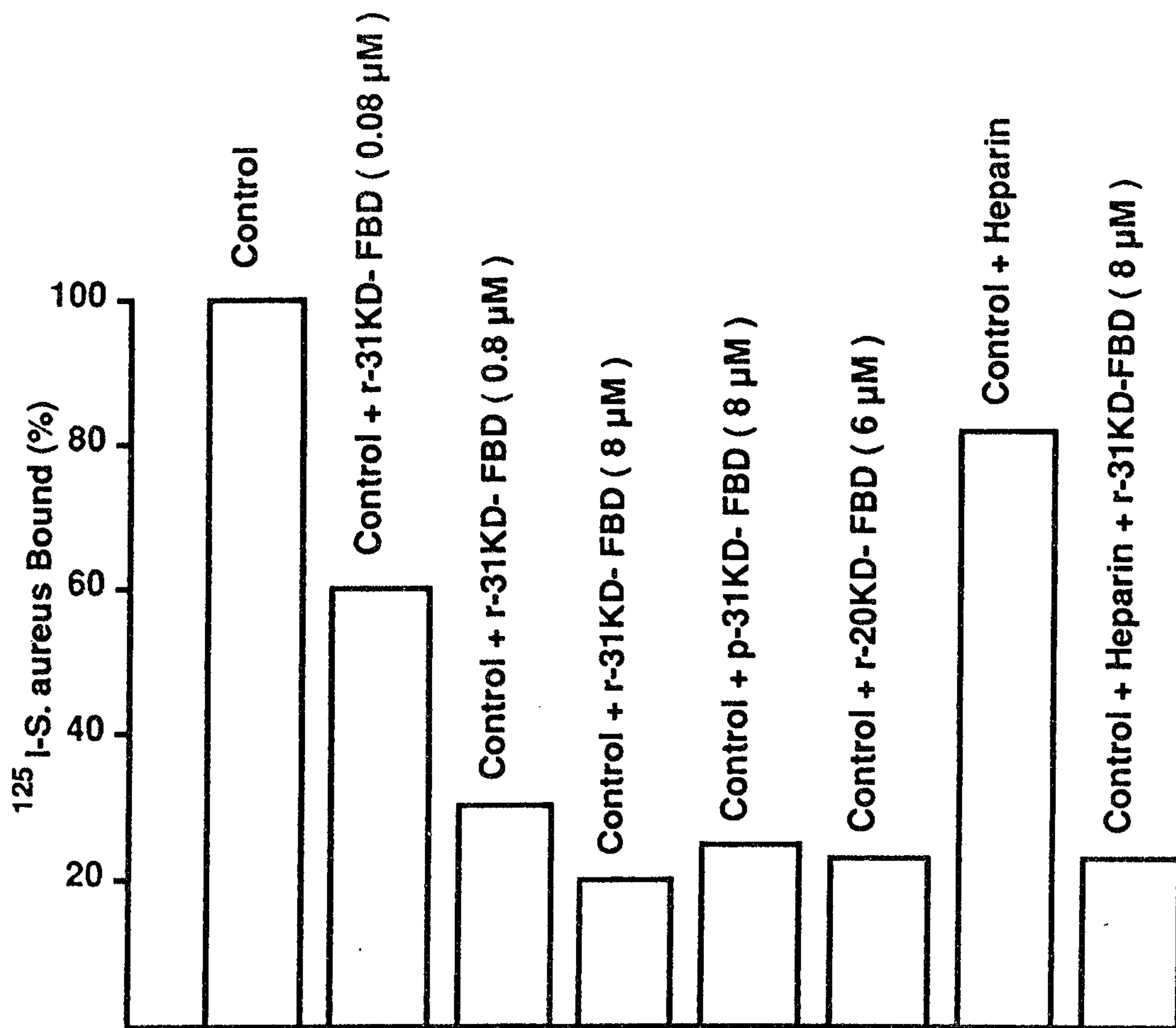
Figure 67

Binding of *S. aureus* to Immobilized FN.

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Figure 68

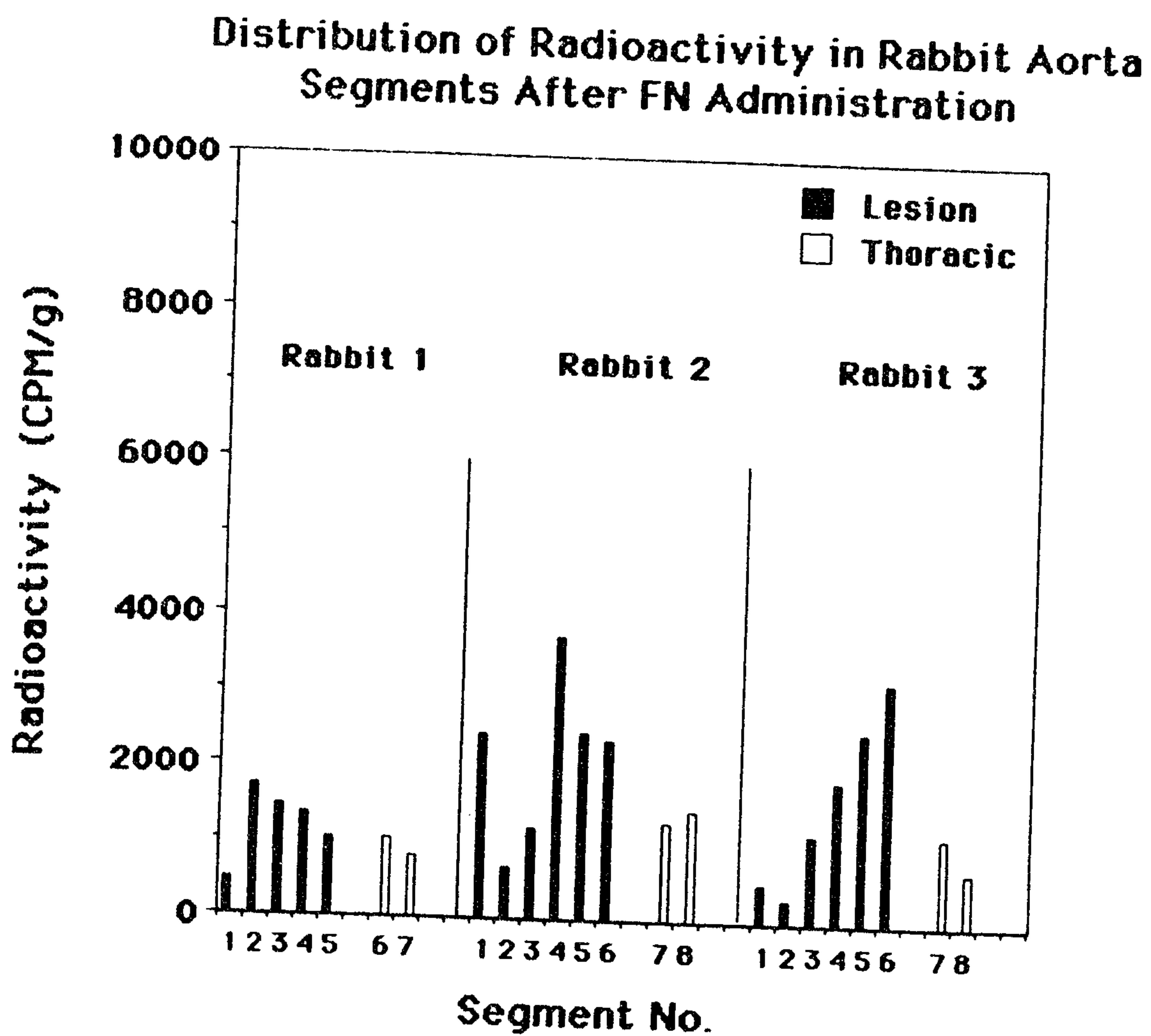
Binding of *S. aureus* to Bronchial Catheters;
Effect of FBD and Heparin.



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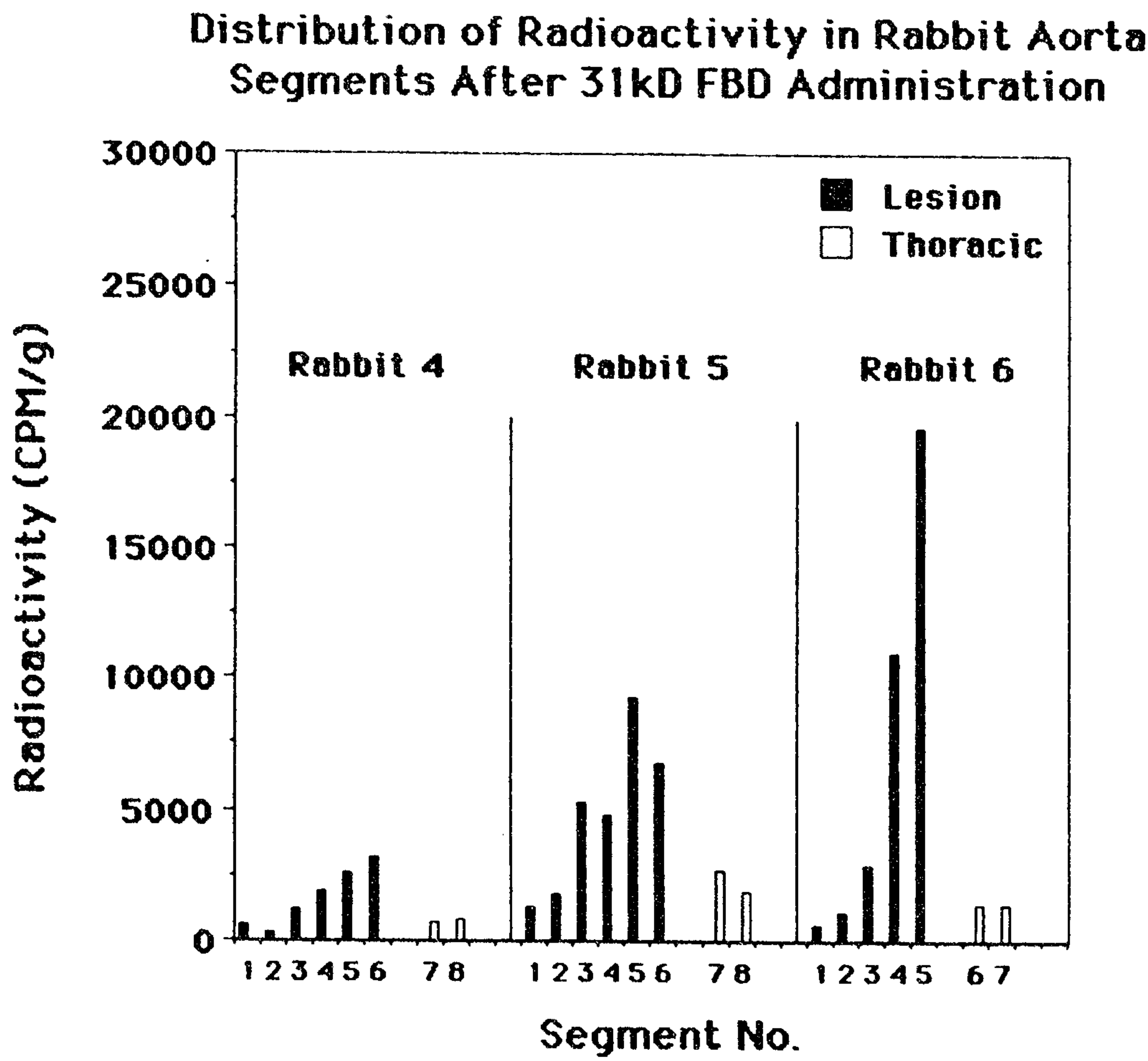
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Figure 69A

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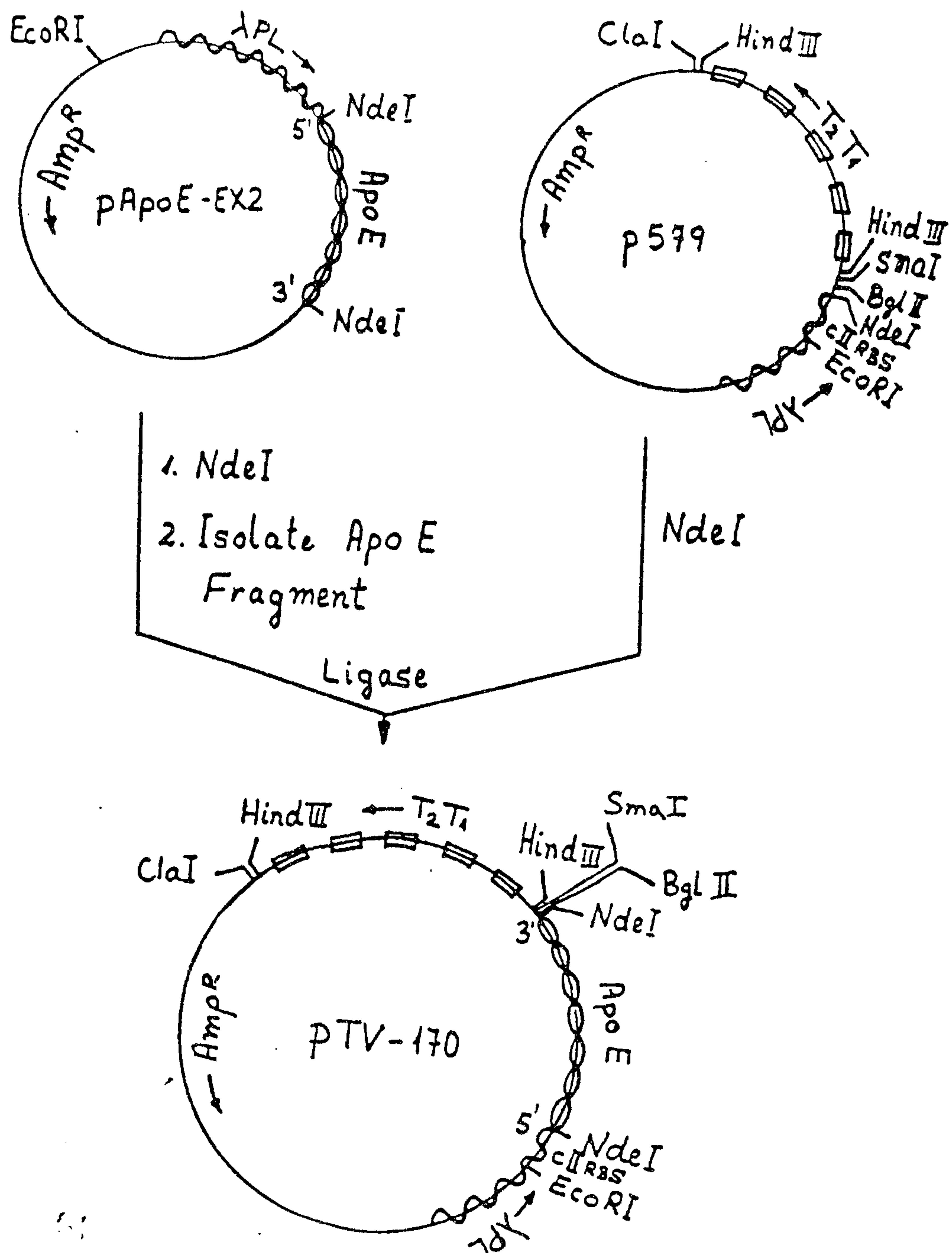
Figure 69B



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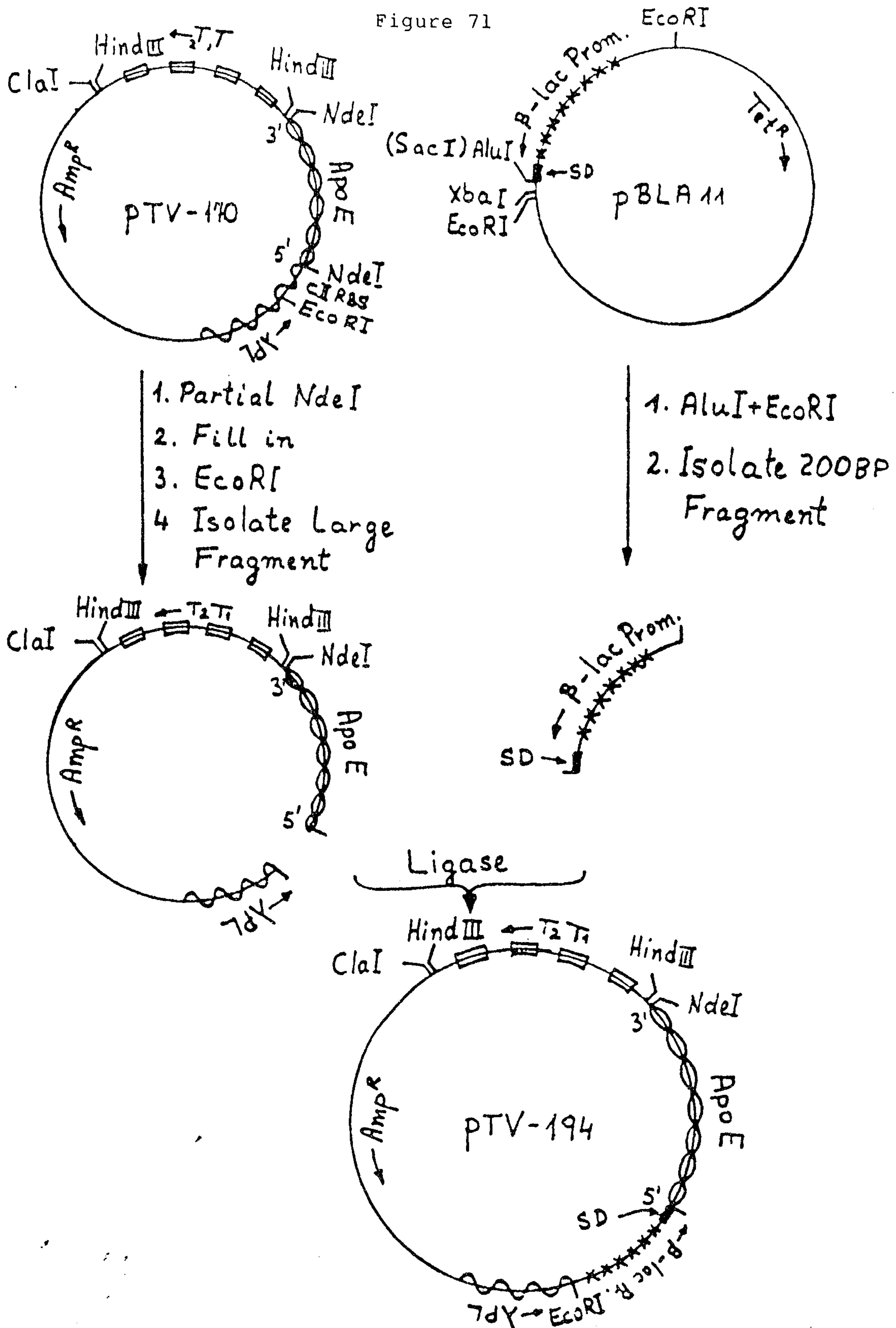
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Figure 70



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Figure 71



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