



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 14/47, G01N 33/68</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/19955</b> <b>(43) International Publication Date:</b> 5 June 1997 (05.06.97)
<b>(21) International Application Number:</b> PCT/US96/18878 <b>(22) International Filing Date:</b> 26 November 1996 (26.11.96) <b>(30) Priority Data:</b> 08/564,526      29 November 1995 (29.11.95)      US <b>(71) Applicant:</b> DADE INTERNATIONAL INC. [US/US]; 1717 Deerfield Road, Deerfield, IL 60015 (US). <b>(72) Inventors:</b> MORJANA, Nihmat, A.; 730 S.W. 99 Avenue, Pembroke, FL 33025 (US). DEMARCO, Curtis; 13510 N.W. 6th Drive, Plantation, FL 33325 (US). <b>(74) Agents:</b> WINSTON, Lois, K. et al.; 1717 Deerfield Road, Deerfield, IL 60015 (US).	<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>	
<b>(54) Title:</b> HUMAN HEART CNBr TROPONIN I ISOFORM AND USE OF SAME		
<b>(57) Abstract</b>  <p>This invention relates to a human cardiac Troponin I fragment generated from human recombinant Troponin I by chemical cleavage. The fragment represents 73 % of the primary structure of human cardiac Troponin I and is immunologically more reactive than recombinant Troponin I. The fragment, or isoform, is comparable in molecular weight to a major degradation product of native cardiac Troponin I in the serum of patients who have experienced myocardial infraction. The isoform can be used as a calibrator or control in cardiac Troponin Immunoassays.</p>		

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**HUMAN HEART CNBr TROPONIN I ISOFORM AND USE OF SAME****Background of the Invention**

This invention relates to the field of the diagnosis of Myocardial Infarction ("MI"). One biochemical test for aiding the diagnosis of MI is the MB isoenzyme of creatine kinase ("CK-MB"). However, CK-MB is not completely  
5 specific for cardiac muscle; it can also be found in skeletal muscle and in blood after skeletal muscle injury. See, e.g., Cummins, et al. (1987), "Cardiac-Specific Troponin I Radioimmunoassay in the Diagnosis of Acute  
10 Myocardial Infarction", American Heart Journal, June 1987, Vol. 113, No. 6. Another disadvantage of the CK-MB test is that the amount of CK-MB in the skeletal muscle varies with the degree of skeletal muscle regeneration, information which may often not be known when administering a test or  
15 analyzing a test result for MI. Another disadvantage of the CK-MB test is that CK-MB remains elevated for only 2-3 days after the onset of chest pain. For patients admitted after that time, the CK-MB test will be of limited, if any, value. See, e.g., Cummins, et al. (1987). Thus, due to  
20 the lack of specificity of the CK-MB test, and the limited time frame for its use as a diagnostic tool, CK-MB is not the MI test of choice. Other enzyme assays also exist, such as lactate dehydrogenase (LDH) and glutamic oxaloacetic transaminase (GOT), but the frequent serial  
25 measurements required in the very early hours after the onset of chest pain could present difficulties for an absolute specific diagnosis. See, e.g., Larue, et al. (1992), "New Monoclonal Antibodies as Probes for Human Cardiac Troponin I: Epitopic Analysis With Synthetic  
30 Peptides", Molecular Immunology, Vol. 29, No. 2, pp. 271-278 (1992). Thus, the prior art has recognized the need for an accurate cardiac-specific biological parameter detectable in serum very soon after MI and remaining present for more than 2-3 days after the onset of MI.

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The cardiac isotype of the myofibrillar contractile protein, Troponin I ("TnI"), is uniquely located in cardiac muscle. TnI is the inhibitory sub-unit of Troponin, a thin filament regulatory protein complex, which confers calcium sensitivity to the cardiac and striated muscle. The Troponin complex consists of three subunits, Troponin T ("TnT") the tropomyosin binding subunit, Troponin C ("TnC"), the Ca<sup>++</sup> binding subunit and TnI, which inhibits the actomyosin Mg<sup>++</sup>-ATPase. Troponin I exists in three isoforms: two skeletal TnI (fast and slow) isoforms (Molecular Weight = 19,800 daltons) and a cardiac TnI ("cTnI") isoform with an additional 31 residues (human TnI) on the N-terminus resulting in a molecular weight of 23,000 daltons.

Cardiac TnI is found in human serum rapidly (within approximately 4 to 6 hours) following a MI. It reaches a peak level after approximately 18-24 hours and remains at elevated levels in the blood stream for up to 6 to 7 days. Thus, immunoassays which can test for human cTnI are valuable to the medical community and to the public.

It is desirable to use an immunologically reactive human cTnI isoform comparable to that detected in MI patient serum. We found that MI patient serum contains TnI fragment(s) which is the result of the C-terminal processing of cTnI molecule. The high sequence homology found in the C-terminal region between cardiac TnI and skeletal muscle TnI (Larue et al. 1992 Molec. Immunology 29, 271-278, Vallins et al. 1990 FEBS Lett. 270, 57-61, Leszky et al. 1988 Biochemistry 27, 2821-2827) produce TnI antibodies directed against this region having non-cardiac specificity (Larue et al. 1992). Our data and Larue et al. 1992 suggest that most of the known cTnI specific antibodies have their epitopes located approximately in the first 75% of the TnI molecule. Therefore, this portion of the TnI molecule should function as a MI specific cTnI isoform in most immunoassay systems.

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Presently, TnI immunoassays do not use human cTnI. Dade currently sells a cTnI Immunoassay Kit in Europe and U.S.A. using a synthetic peptide in the calibrators and the controls. This product is the Stratus® Cardiac Troponin-I  
5 assay. Native human cTnI is difficult to obtain because of the scarcity of human heart. Moreover, native human cTnI is highly subject to proteolytic degradation during purification. The availability of human recombinant TnI ("r-TnI") can facilitate the production of this cTnI  
10 isoform. The r-TnI, unlike the native human cTnI, can be produced and purified in acceptable quantities. As expressed by Dade, the primary structure of r-TnI contains 226 amino acids (SEQ ID NO: 1); 209 of them represent the TnI sequence (SEQ ID NO: 2). (See Fig. 1.) In addition to  
15 the primary sequence of cTnI (SEQ ID NO: 2), r-TnI has a leading sequence of 8 amino acids (MASMTLWM) on the N-terminal, and a tail sequence of 9 amino acids (PMVHHHHHH) on the C-terminal (SEQ ID NO: 1). (See Fig. 1.) The primary structure of the r-TnI molecule has methionine  
20 residues at positions -7, -4, 0, 153, 154, 200 and 211 (SEQ ID NO: 1). (See Fig. 2.)

It is desirable to use an immunologically reactive human cTnI isoform comparable to that detected in MI patient serum. The availability of r-TnI can facilitate the  
25 production of this cTnI isoform. Moreover, since most of the known TnI antibodies have their epitopes located approximately in the first 75% of the TnI molecule, that portion of the TnI molecule will function as a cTnI isoform in most immunoassays.

### 30 Summary of the Invention

This invention relates to the use of a human cTnI fragment generated from human r-TnI by chemical cleavage. The cleavage of r-TnI by cyanogen bromide (CNBr) results in a major polypeptide of 153 amino acids, hereinafter  
35 referred to as the "CNBr-cTnI isoform" (SEQ ID NO: 3). The CNBr-cTnI isoform represents 73% of the primary structure of human cTnI and is immunologically more reactive than r-

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TnI. The purified CNBr-cTnI isoform has an average of 3-4 times more reactivity than r-TnI and lower non-specific binding, as measured by radial partition immunoassay. As demonstrated in Figure 7 the molecular size of the CNBr-cTnI isoform is comparable in molecular weight to the major degradation product of native cardiac TnI in MI patient serum. The CNBr-cTnI isoform can be used as calibrators or controls in various cTnI immunoassays. The CNBr-cTnI isoform has increased stability over the synthetic peptide currently used in the Dade TnI immunoassay.

This invention also relates to the effect of TnC upon the immunological and biological activity and non-specific binding of the CNBr-cTnI isoform. This invention further relates to the complex formed by the CNBr - cTnI isoform, TnC and TnT.

#### Description of the Drawings

Fig. 1 depicts the alignment of the human cardiac Troponin I amino acid sequences. Single letter code has been used. Other symbols include: (r)=r-TnI (SEQ ID NO: 1); (h)=native human cTnI (SEQ ID NO: 2); (I)=CNBr-cTnI isoform (SEQ ID NO: 3); (cam)=S-carboxyamidomethylcysteine.

Fig. 2 depicts the CNBr cleavage strategy of r-TnI; (M) = methionine.

Fig. 3 shows the activity of r-TnI and CNBr-cTnI isoform in calibrator base as measured with the Stratus® II TnI Immunoassay System.

Fig. 4 depicts the activity of r-TnI and the CNBr-cTnI isoform in human serum as measured with the Stratus® II TnI Immunoassay System.

Fig. 5 shows the non-specific binding/specific binding ratio of r-TnI and the CNBr-cTnI isoform on various tabs as measured with the Stratus® II TnI Immunoassay System. The specific activity of TnI forms was measured on TnI specific antibody tabs. The abbreviations for other (TnI non-specific tabs) tabs are: PSA = prostate specific antigen tabs; CKMB = creatine kinase MB tabs; AFP = alpha fetal

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protein tabs; PAP = prostate acid phosphatase tabs; blank = blank glass fiber tabs; isoform = CNBr-cTnI isoform.

Fig. 6 depicts the results of the SDS - polyacrylamide gel electrophoresis (15%) of the CNBr-cTnI isoform. Lanes 1 and 8 are the molecular weight standard, Lanes 2 and 5 are the r-TnI, and Lanes 3, 4, 6, and 7, the CNBr-cTnI isoform.

Fig. 7 depicts the Western Blot analysis of the CNBr-cTnI isoform. Lane 1 is the CNBr-cTnI fragment, Lanes 2 and 3 are the degradation fragment of cTnI extracted from MI patient serum, and Lane 4 the molecular weight standard.

Fig. 8 shows the alignment of the N-terminal amino acid sequence of the CNBr-cTnI isoform ("c") (SEQ ID NO: 3) with those of human cardiac TnI ("b") (SEQ ID NO: 2) and r-TnI ("a") (SEQ ID NO: 1).

Fig. 9 shows the results of polyacrylamide gel electrophoresis (native gel) of TnI:TnC complexes. Lane 1 is TnC. Lane 2 is r-TnI:TnC (1 mol:1 mol). Lane 3 is rTnI:TnC (2 mol:1 mol). Lane 4 is rTnI:TnC (3 mol:1 mol). Lane 5 is CNBr-cTnI isoform:TnC (1 mol:1 mol). Lane 6 is CNBr-cTnI isoform:TnC (2 mol:1 mol). Lane 7 is CNBr-cTnI isoform:TnC (3 mol:1 mol).

Fig. 10 depicts the activity and non-specific binding of various TnI preparations. The activity of the various TnI forms and their respective complexes was determined using the Stratus® II TnI Immunoassay System. Non-specific binding was determined using Ferritin tabs.

Fig. 11 demonstrates the effect of the presence of TnC on the activity of the CNBr-cTnI isoform as measured by the Stratus® II TnI Immunoassay System.

Fig. 12 compares the polyacrylamide gel electrophoresis (10% PAGE, tris-tricine buffer at pH 8.3) (native gel) results of the complex formation of the CNBr-cTnI isoform:TnC:TnT complex (Lanes 3 and 4), and the CNBr-cTnI isoform:TnC complex (Lanes 1 and 2).

Fig. 13 depicts the polyacrylamide gel electrophoresis (native gel) results of the complex formation of r-TnI:TnC

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complex, the CNBr-cTnI isoform:TnC complex, and TnI  
cIsoform II ("cIsoform II"):TnC complex. Lane 1 is the TnC  
control; Lane 2 is r-TnI:TnC at a 1:1 mol ratio; Lane 3 is  
CNBr-cTnI isoform:TnC at a 1:1 mol ratio; Lane 4 is  
5 cIsoform II:TnC, at a 1:1 mol ratio; Lane 5 is r-TnI:TnC at  
a 1:2 mol ratio; Lane 6 is CNBr-cTnI isoform:TnC at a 1:2  
mol ratio, and Lane 7 is cIsoform II:TnC at a 1:2 mol  
ratio. All samples contain 2 mM CaCl<sub>2</sub> and were incubated  
at room temperature for 30 minutes.

10 Fig. 14 shows the stability of the CNBr-cTnI  
isoform:TnC complex in bovine serum. The stability of the  
CNBr-cTnI isoform (1 ug/ml) and the CNBr-cTnI isoform:TnC  
complex (1:1) were followed at 4°C over three weeks. The  
final concentration of CNBr-cTnI isoform alone and in the  
15 complex is 0.25 ug isoform/ml. Three lots of bovine serum  
were used; H,Q,S represent the serum lots Hyc 2242, Quad  
9058 and Sigma S7140 respectively. The TnI activity was  
measured using the Stratus II TnI Immunoassay system.

20 Fig. 15 depicts the non-specific binding of the CNBr-  
cTnI isoform and the CNBr-cTnI isoform:TnC complex in  
bovine serum. The non-specific binding measurements were  
carried out on the Stratus II Immunoassay system using  
Ferritin tabs. The bovine serum lots H,Q,S represent Hyc  
2242, Quad 9058 and Sigma S7140 respectively.

25 Fig. 16 demonstrates the stability of the CNBr-cTnC  
isoform and the CNBr-cTnC isoform:TnC complex in human  
serum. The final concentration of CNBr-cTnI isoform alone  
and in the complex (1:1) is 0.25 ug/ml. The TnI activity  
was measured on the Stratus II TnI immunoassay system.

30 Fig. 17 shows the non-specific binding of the CNBr-  
cTnI isoform and the CNBr-cTnI isoform:TnC complex in human  
serum. The non-specific binding measurements were carried  
out on the Stratus II Immunoassay system using Ferritin  
tabs and blank tabs.

35 Fig. 18 depicts the stability of the reconstituted  
CNBr-cTnI isoform:TnC complex (1:1) in bovine serum. Two  
lots of bovine serum were used, BTI and Quad, at three

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levels. Measurements were carried out using the Stratus II TnI immunoassay system.

Fig. 19 depicts a Map of expression vector pTac/Gene 10/Troponin I/6x His.

5

#### Detailed Description of the Invention

The recombinant human cTnI was expressed in E. coli by the Dade Biology Skills Center (and we thank the Dade Biology Skills Center for providing it). TnI was cloned from human heart cDNA, which is commercially available through companies such as Strategene, by polymerase chain reaction (PCR) and was subcloned into the NcoI restriction site in the Dade constructed vector pTac 102-2, as shown in Fig. 19. (Purified TnI from bovine or human heart is also commercially available.) The vector pTac 102-2 was constructed by conventional means (see Vallins et al, Molecular cloning of human cardiac troponin I using PCR, FEBS Lett. 270, 57-61 (1990)) to include a HindII-Bam HI fragment containing p<sup>tac</sup>, a strong hybrid promoter driving gene transcription. The promoter was induced in E-coli through IPTG, a method and technique well known to those skilled in the art. The next downstream DNA sequence is an efficient ribosomal binding site (RBS) and the N-terminal five amino acids of gene 10 for translation initiation, followed by the cloning cite NcoI. The Dade designed TnI (amino acids 1-210 (SEQ ID NO: 4), including the initiating methionine) was inserted into NcoI in frame with the N-terminus, and with six Histidine codons at the C-terminus (HIS6). The expressed protein contained 226 amino acids. The HIS6 C-terminus facilitated a single step purification. Cleavage of the r-TnI molecule at the methionine residues at positions -4, 0, 153, 154, 200 and 211 by CNBr produced a major polypeptide of 153 amino acids. (SEQ ID NO: 3.) The resulting polypeptide had 73% of the human cTnI primary structure (209 amino acids) (SEQ ID NO: 3), and retained the epitopes for the antibodies used in the Stratus® II TnI

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Immunoassay System. (See Vallins et al 1990 FEBS Lett. 270, 57-61.)

Cyanogen bromide cleaves at methionine residues with high specificity under acidic conditions. Cleavage of the r-TnI by CNBr at all methionine sites should produce 6 peptides of various sizes

(SEQ ID NO: 5) (SEQ ID NO: 3) (SEQ ID NO: 8)

(SEQ ID NO: 6) (SEQ ID NO: 7) (SEQ ID NO: 9)

(See Fig. 2). Generally described, the first step is to

carboxymethylate the cysteine residues (there are two in the TnI sequence) (SEQ ID NO: 1) at positions 79 and 96 in order to prevent dimerization by inter or intra molecular disulfide bridges. CNBr treatment is carried out on the carboxymethylated r-TnI. Unlike other possible cleavage reactions (e.g. enzymatic), the CNBr treatment removes the tail sequence, the leading sequence, and part of the TnI C-terminal region without affecting the primary sequence of the immunogenic sites.

#### **Example I (Preparation of CNBr-cTnI Isoform)**

Three trial preparations of the CNBr-cTnI isoform were conducted. Recombinant TnI (10-20 mg., 0.25-0.3 mM) in 100 mM Na-phosphate, 10 mM tris, 8 M urea, pH 8 ("PTU buffer") was reduced by adding sufficient Dithiothreitol (DTT) freshly prepared in the same buffer (200 mM stock solution) to give a final concentration of 2.5 mM of DTT.

The mixture was incubated at room temperature (approximately 23-25°C) for a time sufficient to reduce the rTnI (approximately 1 hour). The reduced r-TnI was treated with iodoacetamide (prepared in the PTU buffer, 400 mM stock solution) to give a final concentration of 15 mM of iodoacetamide in the reaction mixture. The mixture was then incubated for a time and under conditions sufficient to complete the carboxymethylation reaction (approximately 1 hour) in the dark at 37°C.

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The mixture was transferred to 10 x 25 mm wide spectra/por(12-14 kd MWCO) dialysis tubing and dialyzed against 2x1L of 25% acetic acid for 24 hours at room temperature, with stirring.

5 The dialyzed cTnI was lyophilized under vacuum (<1 mm Hg) between room temperature and 45°C. The lyophilized rTnI was dissolved in approximately 1.4 ml 70% formic acid and then CNBr (1 ug/uL in 70% formic acid) was added to the rTnI solution to give a final concentration of 480 mM of  
10 CNBr (approximately 160 mol CNBr/mol methionine). The tube containing the reaction mixture was purged with nitrogen and then incubated at least 16 hours at room temperature in the dark with rocking. The reaction was stopped by adding distilled water to give 1:10 dilution of the digest. The  
15 digest was lyophilized under vacuum (<1 mm Hg) between room temperature and 45°C. The lyophilized digest was dissolved in a minimum volume of 88% formic acid. The digest was applied on a Sephadex G-200 (1.6 x 100 cm) column equilibrated with 25% acetic acid. The CNBr-cTnI isoform  
20 was eluted with 25% acetic acid. The first major peak, which represented the CNBr-cTnI isoform, was pooled and tested for purity by SDS-PAGE and for immunoreactivity by the Stratus® II TnI Immunoassay System.

As measured by the Stratus® II TnI Immunoassay System,  
25 the purified CNBr-cTnI isoform has 3-4 fold higher immunological activity than r-TnI (See Figs. 3 and 4.)

The following data are graphed in Fig. 3.

	TnI (Lot)	r-TnI	CNBr-cTnI isoform
	RD 4	853	3872
30	PP 1	949	3816
	PP 2	1148	3720

35 Values are expressed in mv/min, which is a standard unit used to measure rate of change. The r-TnI or CNBr-cTnI isoform was spiked into Calbase in a total volume of 1 ml. TnI concentration was 0.5 mg/ml. The ratio between non-specific binding and specific binding of the CNBr-cTnI

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isoform is lower than that of r-TnI. (See Fig. 5.) The following data are graphed in Fig. 5.

	TnI Forms	Specific Binding	Non-Specific Binding
5	CNBr-cTnI isoform	3426.6	1328.6
	CNBr-CTnI isoform: TnC complex	42,333.6	1890.0
	r-TnI	491.3	334.2
	r-TnI:TnC complex	3749.4	1742.3

10 Values are expressed in mv/min. An aliquot of the r-TnI or CNBr-cTnI isoform was spiked in Calbase to give a final concentration of 20 nM (3.5 ul of CNBr-cTnI isoform 0.1 mg/ml is spiked into 996.5 ul Calbase; 5.2 ul of r-TnI 0.1 mg/ml is spiked into 994.8 ul Calbase).

15 For the complexes with TnC, the reaction mixtures were prepared as follows. For the r-TnI:TnC complex, 5.2 ul r-TnI (0.1 mg/ml) plus 3.65 ul TnC (0.165 mg/ml) plus 2 ul CaCl<sub>2</sub> (25 mM) plus 14.15 ul PTU buffer. The total volume measured 25 ul. The reaction was incubated at room  
20 temperature for approximately 15 minutes and then spiked into 975 ul Calbase. For the CNBr-cTnI isoform: TnC complex, 3.5 ul TnI (0.1 mg/ml) plus 3.65 ul TnC (0.165 mg/ml) plus 2 ul CaCl<sub>2</sub> (25 mM) plus 15.85 ul PTU buffer. The total volume was 25 ul. The reaction was incubated at  
25 room temperature for approximately 15 minutes and then spiked into 975 ul Calbase.

The purified CNBr-cTnI isoform migrates on SDS-PAGE gel electrophoresis as a single band with an apparent molecular weight of 21,000 daltons. Western blot analysis  
30 of the CNBr-cTnI isoform has a molecular weight close to that of a major degradation fragment of cTnI in MI patient serum. (See Fig. 7) The N-terminal sequence analysis of the isoform gave the sequence Ala-Asp-Gly-Ser-Ser-Asp-Ala-Ala-Ala-Arg-Glu, which is identical to the N-terminal  
35 sequence of human cTnI (SEQ ID NO: 2)(See Fig. 8). The amino acid analysis (See Table 1) confirms that the purified CNBr-cTnI isoform represents the first 153 amino acids of the cTnI molecule. (SEQ ID NO: 1.) Rabbit

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skeletal muscle TnC was purified as described by Potter, J.D. (1982) in Methods Enzymology 85, 241-263. TnC from other tissue sources can be used as well. TnT was obtained commercially.

5           The CNBr treatment proved specific, with no evidence of side or non-specific reactions. Other chemical and proteolytic means lack specificity and the experimental conditions are hard to control. Even using very specific proteolytic enzymes, the possibility is quite high of  
10 affecting the epitopes of various cTnI antibodies including those used in the Stratus® TnI Immunoassay System. Another procedure, complicated and costly, would be to make the 153 amino acid cTnI isoform (SEQ ID NO: 3) through cDNA and expression in E. coli or other expression systems after  
15 changing the Cys codons if desired. The carboxymethylation of the cysteine residues is not a pre-requisite for the generation of the 153 amino acid isoform. (SEQ ID NO: 3.) Rather, the carboxymethylation facilitates the process by minimizing the complications during or after CNBr  
20 digestion.

          Using the CNBr cleavage procedure, the CNBr-cTnI isoform can only be generated from human cTnI or human r-TnI. The CNBr cleavage of cTnI from other species (bovine, rabbit, etc.) does not generate the 153 amino acid isoform  
25 because in such species, the non-human cTnI has a methionine residue at position 53 in the amino acid sequence, which would also undergo the cleavage reaction with CNBr. In human cTnI or r-TnI, leucine replaces the methionine at position 53.

30           Longer or shorter form(s) of the CNBr-cTnI isoform can be produced by adding or deleting a few amino acids to/from the N terminal, the C-terminal or any part of the TnI isoform sequence. Human cTnI cDNA cloned into a vector can be modified by site directed mutagenesis (oligonucleotides)  
35 and/or PCR (Guo et al., 1994 J. Biol. Chemistry 269, 15210-15216, Farah et al. 1994 J. Biol. Chem. 269, 5230-5240, Sheng et al. 1992 J. Biol. Chem. 267, 25407-25413) to

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produce CNBr-cTnI isoform or its modified forms. The modified cDNA can be subcloned into a vector to give rise to the expression construct for CNBr-cTnI isoform or its modified form(s). The protein expression can be carried  
5 out in E. Coli or other expression system. Changes in some amino acids of the CNBr-cTnI isoform sequence might not affect its performance except those occurring at the epitope(s) where the specific assay antibodies bind.

The buffer used for the carboxymethylation of r-TnI  
10 can be replaced by other buffers with a pH of about 8. Dithiothreitol can be substituted with other reducing agents, particularly those which are effective and work maximally at a pH of around 8, such as glutathione, DTE, acetyl cysteine. Alkylating reagents other than  
15 iodoacetamide can be used (e.g. iodoacetic acid, NEM, etc.) in order to block cysteine residues. The time needed for the cleavage of TnI by CNBr could vary between 10-24 hours at room temperature in the dark. The CNBr cleavage must be carried out under acidic conditions, because the  
20 selectivity of the reaction of CNBr with amino acids depends on pH. Acids other than formic acid, such as trifluoroacetic acid, can be used. The method of purification of the isoform is not critical. It may be purified by various chromatographic methods. Size  
25 exclusion columns such as the Sephacryl S-200, Separose 12, and Sephadex G 100, 150 and 200, are useful at large scale. The isoform can also be purified by TnC affinity column such as TnC-sepharose affinity column, as well as other TnC affinity columns.

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**Example II (Binding Properties of the CNBr-cTnI Isoform with TnC)**

TnI binds TnC in the presence of calcium ions. For complete formation of the CNBr-cTnI:TnC complex at least  
35 one mole of TnC is needed per mole of the CNBr-cTnI isoform. The time required to form the complex is flexible. The CNBr-cTnI isoform is able to form a complex

- 13 -

with TnC from various species. The complex can be formed best at pH range of 4-8.5 in the absence or presence of urea. The binding properties of the CNBr-cTnI isoform with TnC have been studied using polyacrylamide gel electrophoresis (native gel). The CNBr-cTnI isoform was incubated with TnC at molar ratios of 1:1, 2:1 and 3:1 in PTU buffer (100 mM Na-phosphate, 10 mM tris buffer containing 8 M urea pH 8). All samples were incubated for 30 minutes at room temperature in the presence of up to 2 mM CaCl<sub>2</sub>. (A shorter time, such as 15 minutes, or possibly less, may also be sufficient.) As depicted in Figure 9, Lane 6, one mole CNBr-cTnI isoform is required to bind one mole of TnC. The formation of the CNBr-cTnI isoform:TnC complex is accompanied by the disappearance of TnC (Lane 6). However, as depicted in Figure 9, at least two moles of r-TnI is required to bind one mole TnC for the formation of the r-TnI:TnC complex (Lane 4). These results suggest that the CNBr-cTnI isoform binds TnC more efficiently than does r-TnI.

**Example III (Immunological Activity of the CNBr-cTnI Isoform:TnC complex)**

The immunological activity of the CNBr-cTnI isoform:TnC complex was measured using the Stratus® TnI Immunoassay System. The CNBr-cTnI isoform was incubated with TnC in PTU buffer at a molar ratio of (1:1) in the presence of 2 mM CaCl<sub>2</sub> for 30 minutes at room temperature. The complex was then added to calibrator base to give a final TnI concentration of 20 nM. (The calibrator base used to measure the complex contains no EDTA but has a concentration of 2 mM CaCl<sub>2</sub>.) CNBr-cTnI isoform alone was added to calibrator base to give a final concentration of the CNBr-cTnI isoform of 20 nM and incubated separately. Similarly, r-TnI was incubated with TnC at a molar ratio of (1:1) in the presence of 2 mM CaCl<sub>2</sub> for 30 minutes at room temperature. The complex was then added to calibrator base to give a final TnI concentration of 20 nM. Recombinant

- 14 -

TnI alone was added to calibrator base to give a final TnI concentration of 20 nM and incubated separately. As shown in Fig. 10, binding the CNBr-cTnI isoform to TnC in the presence of calcium ions enhances the activity of the CNBr-cTnI isoform several times over that of the CNBr-cTnI isoform alone. The non-specific binding of the CNBr-cTnI isoform:TnC complex is low, less than 5% of the total activity. In comparison, binding of r-TnI to TnC increases its activity but not nearly to the extent observed with the CNBr-cTnI isoform:TnC complex.

The effect of TnC on the activity of the CNBr-cTnI isoform was examined using the Stratus® II TnI Immunoassay System. The CNBr-cTnI isoform was incubated with TnC in PTU buffer containing 2 mM CaCl<sub>2</sub> at room temperature for 30 minutes. The CNBr-cTnI isoform:TnC complex was prepared at ratios of 1:4, 1:2, 1:1, 1:0.5, 1:0.25 and 1:0.00 (mol/mol). An aliquot of each reaction mixture was spiked into calibrator base (without EDTA, but containing 2 mM CaCl<sub>2</sub>) to give a final concentration of 6.5 nM of CNBr-cTnI isoform. Measurements were carried out on the Stratus® II Immunoassay System. As shown in Fig. 11, the 1:1 ratio of CNBr-cTnI isoform:TnC complex produces maximum activity. Addition of more TnC slightly increases the TnI activity, but has no effect on the non-specific binding of the complex. Fig. 9 shows that at the 1:1 ratio, the CNBr-cTnI isoform is totally complexed with TnC.

**Example IV (Reconstitution of Troponin Complex using CNBr-cTnI isoform)**

The complex between the CNBr-cTnI isoform, TnC and TnT was formed by mixing stoichiometric amounts of each subunit in 100 mM sodium phosphate buffer pH 7.5 containing 10 mM tris, 1 mM CaCl<sub>2</sub>, 7 mM mercaptoethanol and 4 M urea. The mixture was incubated at room temperature for 3 hours and then either used for analysis or dialyzed into the buffer of interest for storage. Formation of the CNBr-cTnI isoform:TnC:TnT complex was examined by using

- 15 -

polyacrylamide gel electrophoresis (native gel). Figure 12 shows that the CNBr-cTnI isoform:TnC:TnT complex, in Lanes 3 and 4, has different mobility than the CNBr-cTnI isoform:TnC complex in Lanes 1 and 2. The activity of the CNBr-cTnI isoform:TnC:TnT complex was measured using the Stratus® II TnI Immunoassay System. The activity of the CNBr-cTnI isoform:TnC:TnT complex is several times higher than that of the CNBr-cTnI isoform and comparable to the activity of the CNBr-cTnI isoform:TnC complex. These results suggest that the CNBr-cTnI isoform is able to form a ternary complex with both TnC and TnT subunits, a property of the TnI molecule. Further, the results suggest that the addition of TnT to the CNBr-cTnI isoform:TnC complex does not interfere with the CNBr-cTnI isoform activity on the Stratus II TnI Immunoassay System. The results also suggest that the high TnI activity of the CNBr-cTnI isoform:TnC:TnT complex is due to the binding to TnC.

**Example V (Stability of the CNBr cTnI Isoform:TnC Complex)**

The effect of the presence of TnC on the stability of the CNBr-cTnI isoform in serum was studied. The presence of TnC was found to increase the stability of the CNBr-cTnI isoform. The CNBr-cTnI isoform and TnC were incubated at a 1:1 ratio in PTU buffer containing 2 mM CaCl<sub>2</sub> for 30 minutes. The stability was studied in 3 lots of bovine serum obtained from three different vendors (Hyc 2242, Quad 9058 and Sigma S7140) (Fig. 14), a human serum pool (Fig. 15) and followed over a three week period. Once added to the serum, the temperature was kept at 4°C. The final concentration of the CNBr-cTnI isoform and the CNBr-cTnI isoform:TnC complex, in serum, was 1 ug/ml and 0.25:0.25 ug/ml respectively.

Fig. 16 shows the stability of the CNBr-cTnI isoform:TnC complex (1:1) spiked into serum at three distinct levels (manufacturing ranges), lyophilized in

- 16 -

small vials (3ml), and stored at 4°C until use. The lyophilized preparations of the CNBr-TnI isoform:TnC complex were reconstituted and assayed at time intervals as shown in Fig. 16.

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**Example VI (Non-specific binding of the CNBr-cTnI isoform:TnC Complex)**

We have studied the effects of the presence of TnC on the non-specific binding of the CNBr-cTnI isoform in serum. Fig. 5 shows that the CNBr-cTnI isoform has lower non-specific binding than r-TnI. As demonstrated in Fig. 17, the non-specific binding of the CNBr-cTnI isoform:TnC complex in bovine serum was lower than that of the CNBr-cTnI isoform. Similar results were also obtained in human serum (Fig. 18).

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**Example VII (Preparation of and Properties of a TnI 88 Amino Acid--cIsoform II)**

cIsoform II was generated from r-TnI (see Fig. 1) using the endoproteinase Asp-N ("EndoAsp"). EndoAsp, a metalloprotease, cleaves at the N-terminus of aspartic acid. Recombinant TnI was incubated with EndoAsp at a ratio of 100:1 (r-TnI:EndoAsp, w/w) in 50 mM sodium phosphate pH 8 containing 1 M urea for 20 hours at 37°C. The major cleavage product consisted of 88 amino acids starting at position 6 (Aspartic acid, D) and ending at position 95 (Glutamine, Q) (Fig. 1) (SEQ ID NO: 10). Once purified, the cIsoform II was tested for purity and activity on SDS-polyacrylamide gel electrophoresis and the Stratus® II TnI Immunoassay System, respectively. As shown in Figure 10, the cIsoform II has higher activity (2-fold) and higher non-specific binding (2-fold) than the CNBr-cTnI isoform. However, little enhancement in TnI activity of cIsoform II was seen when it was incubated with TnC in the presence of 2 mM CaCl<sub>2</sub>.

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The ability of the cIsoform II to form a complex with TnC was examined. Fig. 13 shows that the binding between the cIsoform II and TnC is weaker than that between the CNBr-cTnI isoform and TnC. A faint band representing the cIsoform II:TnC complex is seen in Lane 4. The majority of the TnC remains unbound. The addition of an excess of TnC does not increase the cIsoform II:TnC complex formation. The results suggest that the minimal effect of TnC on TnI activity of the cIsoform II is due to the inability of the cIsoform II to form a stable complex with TnC. Fig. 13, Lane 3, shows that the CNBr-cTnI isoform effectively binds TnC with better efficiency than the r-TnI and cIsoform II.

**Example VIII (The CNBr-cTnI Isoform as a Calibrator and a Control)**

**Preparation of control:** A stock solution of the CNBr-cTnI isoform (1 mg/ml) is prepared in 100 mM sodium phosphate buffer pH 8 containing 10 mM tris and 8 M urea using polypropylene tubes. A liquid tri-level assay control containing the CNBr-cTnI isoform is prepared in serum, diluted serum, plasma, diluted plasma or base using plastic labware. The levels of controls are:

25	Manufacturing <u>Ranges</u>	CNBr-cTnI isoform <u>range (ng/ml)</u>
	Level 1	3 - 6
	Level 2	17-22
	Level 3	35-44

CNBr-cTnI isoform is spiked into serum, diluted serum, plasma, diluted plasma or base at the designated level. The mixtures are filtered and tested on the Stratus® II Immunoassay System for the matching level of TnI concentration. Preparations of each level are placed (3 ml each) in plastic vials which are either stored at 4° C or lyophilized. The lyophilized material is reconstituted using 3 ml of water upon use.

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**Preparation of Calibrators:** The calibrators can be made by adding a sufficient amount of CNBr-cTnI isoform stock solution to serum, plasma or base, to give final concentrations ranging from 2 to 50 ng/ml. The CNBr-cTnI isoform calibrator concentrations are 0 ng/ml, 2 ng/ml, 8  
5 ng/ml, 15 ng/ml, 25 ng/ml and 50 ng/ml. Each calibrator level is filtered and analyzed on the Stratus® II Immunoassay System using TnI immunoassay and matched against the reference level. The calibrators are then  
10 filled into their designated vials and either lyophilized or stored at 4°C. The lyophilized calibrators are reconstituted to the pre-lyophilization volume using water.

The Examples provided herein are for the purposes of illustration only and are not intended to limit the scope  
15 of the invention.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (I) APPLICANTS: Dade International Inc.  
Morjana, Nihmat A.  
DeMarco, Curtis
- (ii) TITLE OF INVENTION: HUMAN HEART CNBr TROPONIN I ISOFORM  
AND USE OF SAME
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: DADE INTERNATIONAL INC.
  - (B) STREET: 1717 Deerfield Road
  - (C) CITY: Deerfield
  - (D) STATE: Illinois
  - (E) COUNTRY: US
  - (F) ZIP: 60015
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 26 November 1996
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/564,526
  - (B) FILING DATE: 29 November 1995
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: WINSTON, Lois K.
  - (B) REGISTRATION NUMBER: 39,074
  - (C) REFERENCE/DOCKET NUMBER: DA-5250
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (708) 267-5364
  - (B) TELEFAX: (708) 267-5376

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 226 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear



## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro Ala  
 1 5 10 15  
 Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu Pro  
 20 25 30  
 His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln Leu  
 35 40 45  
 Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu Ala  
 50 55 60  
 Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys Gln  
 65 70 75 80  
 Pro Leu Glu Leu Thr Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu Cys  
 85 90 95  
 Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr Asp  
 100 105 110  
 Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu Thr  
 115 120 125  
 Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu Arg  
 130 135 140  
 Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly Ala  
 145 150 155 160  
 Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val Lys  
 165 170 175  
 Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg Lys  
 180 185 190  
 Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe Glu  
 195 200 205

Ser

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 153 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear



Gln Pro Leu Glu Leu Thr Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu  
 85 90 95

Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr  
 100 105 110

Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu  
 115 120 125

Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu  
 130 135 140

Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly  
 145 150 155 160

Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val  
 165 170 175

Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg  
 180 185 190

Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe  
 195 200 205

Glu Ser Pro  
 210

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 3 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Ser Met  
 1

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 4 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Thr Leu Trp Met  
 1

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 46 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gln Ala Leu Leu Gly Ala Arg Ala Lys Gly Ser Leu Asp Leu Arg Ala  
 1                                   5                                   10                                   15  
 His Leu Lys Gln Val Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu  
                                   20                                   25                                   30  
 Val Gly Asp Trp Arg Lys Asn Ile Asp Ala Leu Ser Gly Met  
                                   35                                   40                                   45

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 11 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Glu Gly Arg Lys Lys Lys Phe Glu Ser Pro Met  
 1                                   5                                   10

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 7 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Val His His His His His His  
 1                                   5

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 88 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro Ala Pro Ile Arg Arg Arg  
1 5 10 15

Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu Pro His Ala Lys Lys Lys  
20 25 30

Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln Leu Lys Thr Leu Leu Leu  
35 40 45

Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu Ala Glu Glu Arg Arg Gly  
50 55 60

Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys Gln Pro Leu Glu Leu Thr  
65 70 75 80

Gly Leu Gly Phe Ala Glu Leu Gln  
85





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6. The control of claim 5 further comprising a known amount of Troponin C.
7. A peptide derived from native or recombinant human cardiac TnI having immunological activity to antibodies against TnI, said peptide made by the process comprising:
- a) reducing human cardiac Troponin I;
  - b) cleaving the human cardiac Troponin I of step b) with CNBr; and
  - c) recovering the resulting human cardiac Troponin I peptide.
8. A calibrator for a TnI immunoassay comprising: a peptide made by the process of claim 7 and serum or calibrator base.
9. The calibrator of claim 8 further comprising: a known amount of Troponin C.
10. A control for a TnI immunoassay comprising: a peptide made by the process of claim 7 and serum or calibrator base.
11. The control of claim 10 further comprising: a known amount of Troponin C.

**FIG. 1**

**Amino Acid Sequence of TnI Forms**

```

-7          -1 0 1
(r)  M-A-S-M-T-L-W-M-A-D-G-S-S-D-A-A-R-E-P-R-P-A-P-A-P-
(h)          A-D-G-S-S-D-A-A-R-E-P-R-P-A-P-A-P-
(I)          A-D-G-S-S-D-A-A-R-E-P-R-P-A-P-A-P-

I-R-R-R-S-S-N-Y-R-A-Y-A-T-E-P-H-A-K-K-K-S-K-I-S-A-S-R-K-
I-R-R-R-S-S-N-Y-R-A-Y-A-T-E-P-H-A-K-K-K-S-K-I-S-A-S-R-K-
I-R-R-R-S-S-N-Y-R-A-Y-A-T-E-P-H-A-K-K-K-S-K-I-S-A-S-R-K-

L-Q-L-K-T-L-L-L-Q-I-A-K-Q-E-L-E-R-E-A-E-E-R-R-G-E-K-G-R-
L-Q-L-K-T-L-L-L-Q-I-A-K-Q-E-L-E-R-E-A-E-E-R-R-G-E-K-G-R-
L-Q-L-K-T-L-L-L-Q-I-A-K-Q-E-L-E-R-E-A-E-E-R-R-G-E-K-G-R-

A-L-S-T-R-C-Q-P-L-E-L-T-G-L-G-F-A-E-L-Q-D-L-C-R-Q-L-H-
A-L-S-T-R-C-Q-P-L-E-L-T-G-L-G-F-A-E-L-Q-D-L-C-R-Q-L-H-
A-L-S-T-R-C-Q-P-L-E-L-T-G-L-G-F-A-E-L-Q-D-L-C-R-Q-L-H-

          |                               |
          cam                           cam

A-R-V-D-K-V-D-E-E-R-Y-D-I-E-A-K-V-T-K-N-I-T-E-I-A-D-L-
A-R-V-D-K-V-D-E-E-R-Y-D-I-E-A-K-V-T-K-N-I-T-E-I-A-D-L-
A-R-V-D-K-V-D-E-E-R-Y-D-I-E-A-K-V-T-K-N-I-T-E-I-A-D-L-

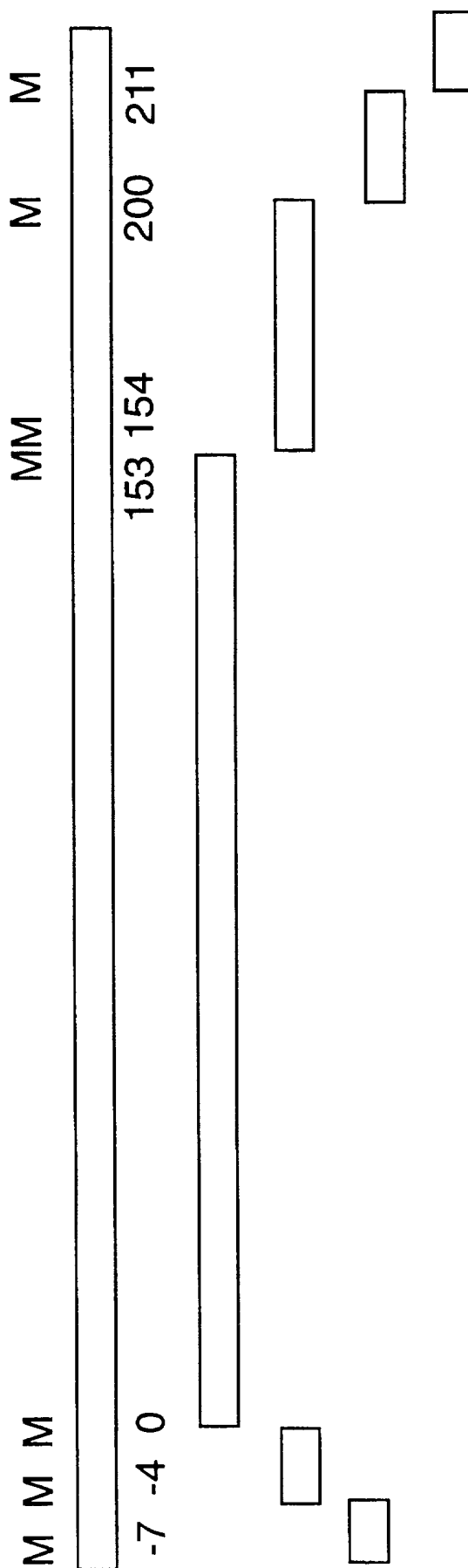
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T-Q-K-I-F-D-L-R-G-K-F-K-R-P-T-L-R-R-V-R-I-S-A-D-A-M-M-
T-Q-K-I-F-D-L-R-G-K-F-K-R-P-T-L-R-R-V-R-I-S-A-D-A-M-

Q-A-L-L-G-A-R-A-K-E-S-L-D-L-R-A-H-L-K-Q-V-K-K-E-D-T-E-
Q-A-L-L-G-A-R-A-K-E-S-L-D-L-R-A-H-L-K-Q-V-K-K-E-D-T-E-

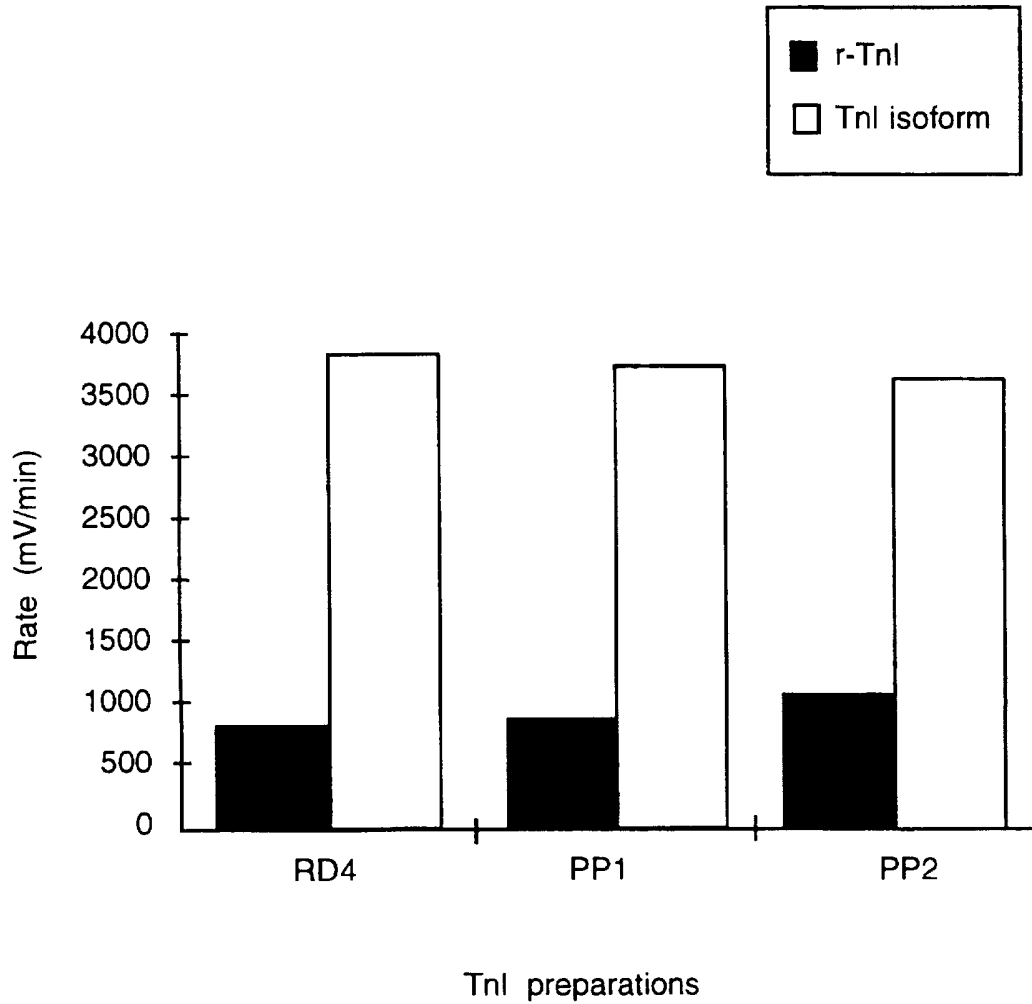
K-E-N-R-E-V-G-D-W-R-K-N-I-D-A-L-S-G-M-E-G-R-K-K-K-F-
K-E-N-R-E-V-G-D-W-R-K-N-I-D-A-L-S-G-M-E-G-R-K-K-K-F-
    210
    |
E-S-P-M-V-H-H-H-H-H-H
E-S
    |
    209

```

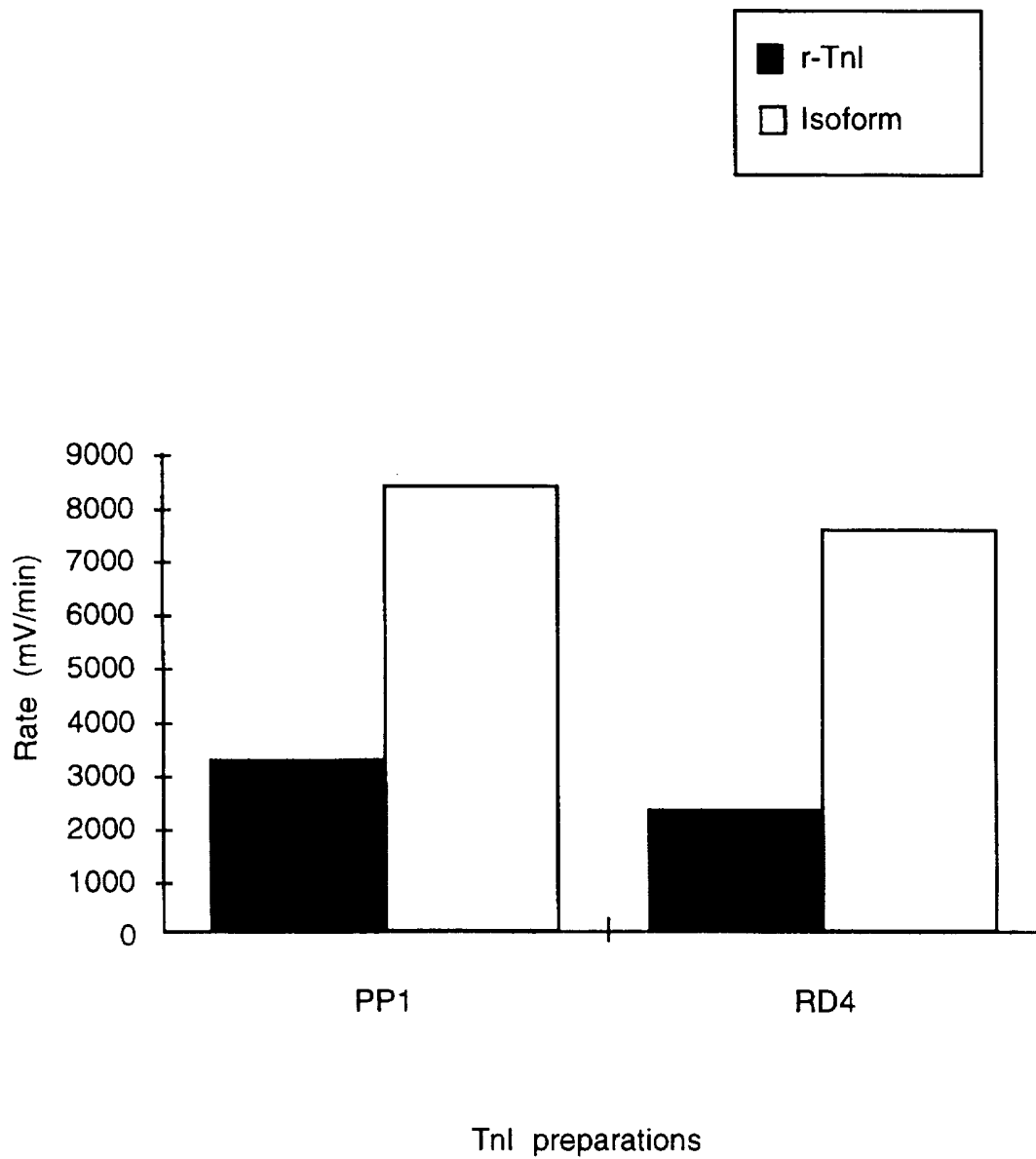
# r-Tnl cleavage strategy



**FIG. 2**

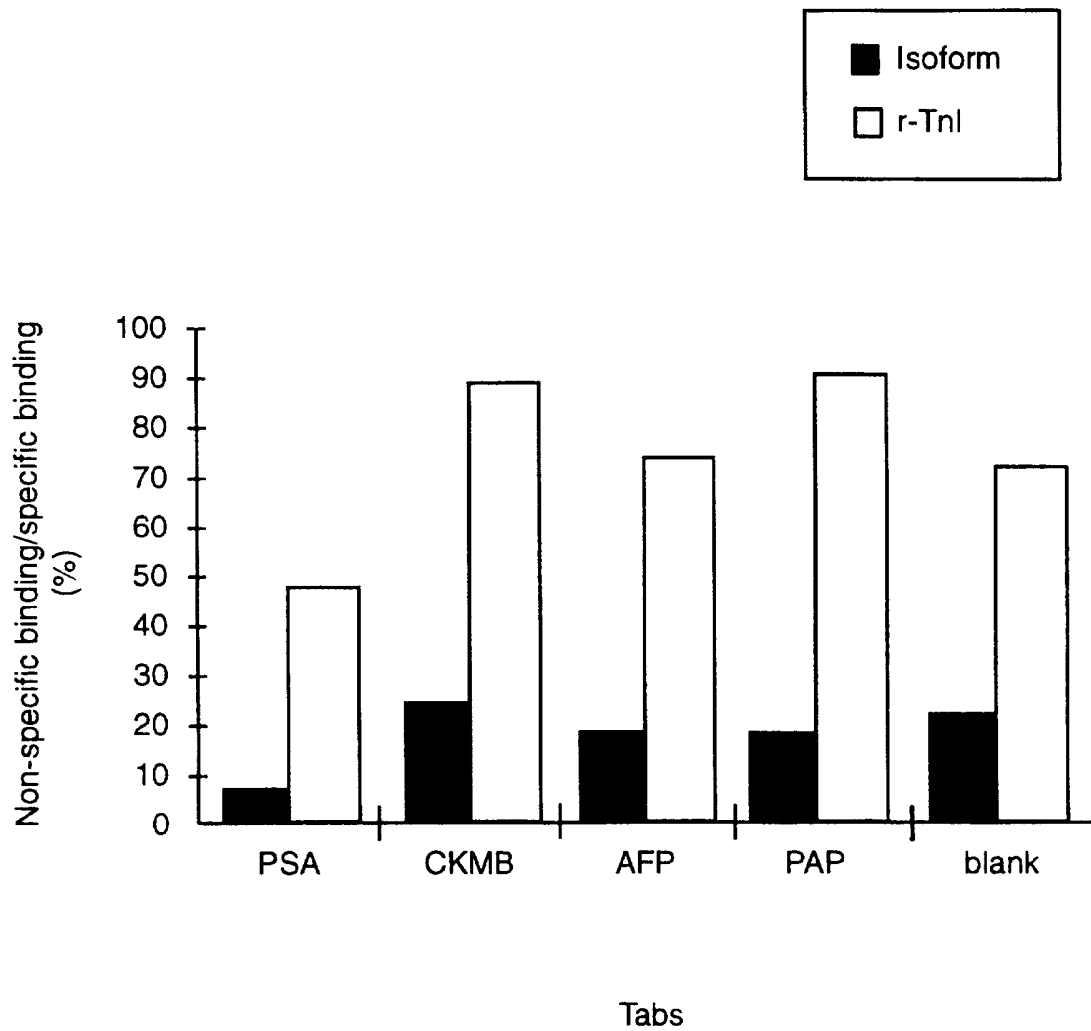


**FIG. 3**

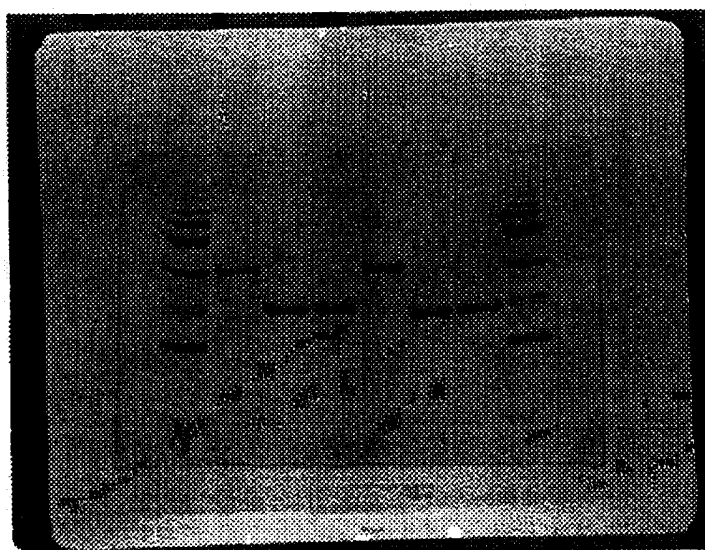


**FIG. 4**

5/20



**FIG. 5**

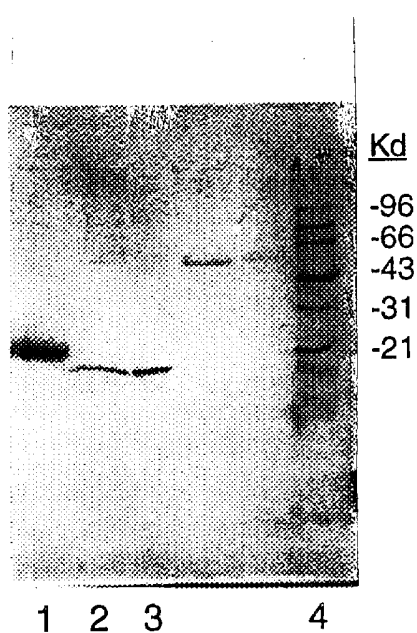


Kd

-96  
-66  
-43  
-31  
-21

1 2 3 4 5 6 7 8

**FIG. 6**



**FIG. 7**

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**FIG. 8****Amino-Terminal Sequence of Tnl Isoforms**

- (a) Met-Ala-Ser-Met-Thr-Leu-Trp-Met-Ala-Asp-Gly-Ser-Ser-Asp-Ala-Ala-Arg-Glu
- (b) Met-Ala-Asp-Gly-Ser-Ser-Asp-Ala-Ala-Arg-Glu
- (c) Ala-Asp-Gly-Ser-Ser-Asp-Ala-Ala-Arg-Glu

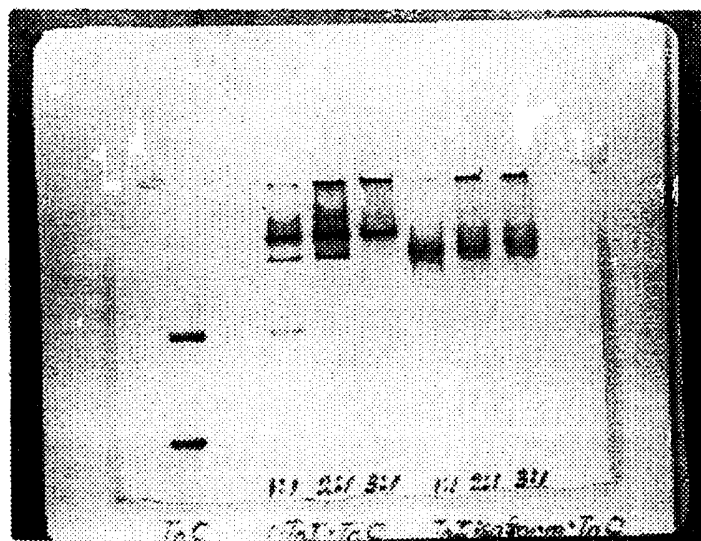
- a- The N-terminal sequence of the recombinant Tnl
- b- The N-terminal sequence of human Tnl (Vallins et al. 1990)
- c- N-terminal analysis of the CNBr Tnl isoform

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**Table 1****Amino Acid Composition of the CNBr Tnl Isoform**

Amino acids	Number of residues per molecule	Number of Residues per molecule from Tnl sequence
Asparatic Acid	10.85	11
Glutamic Acid	21.17	21
Serine	8.54	9
Glycine	6.54	6
Histidine	2.14	2
Arginine	19.73	20
Threonine	6.70	8
Alanine	18.24	18
Proline	7.41	7
Tyrosine	2.81	3
Valine	4.14	4
Methionine	0.82	1
Isoleucine	8.43	8
Leucine	15.78	16
Phenylalanine	2.98	3
Lysine	13.32	14
Cysteic Acid *	-----	2

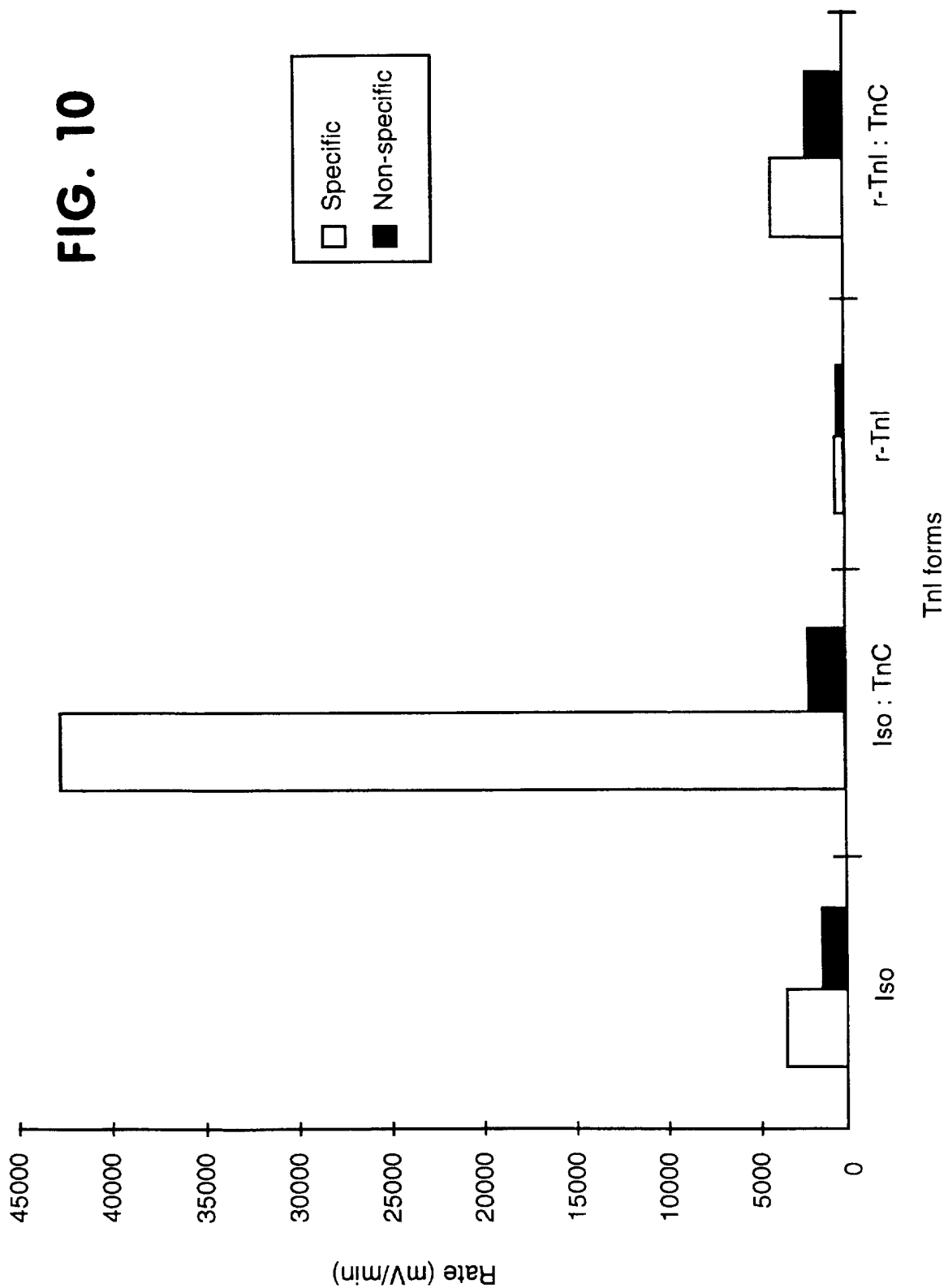
\* The two cysteine residues in the primary sequence of the isoform are blocked (see methods, part III)



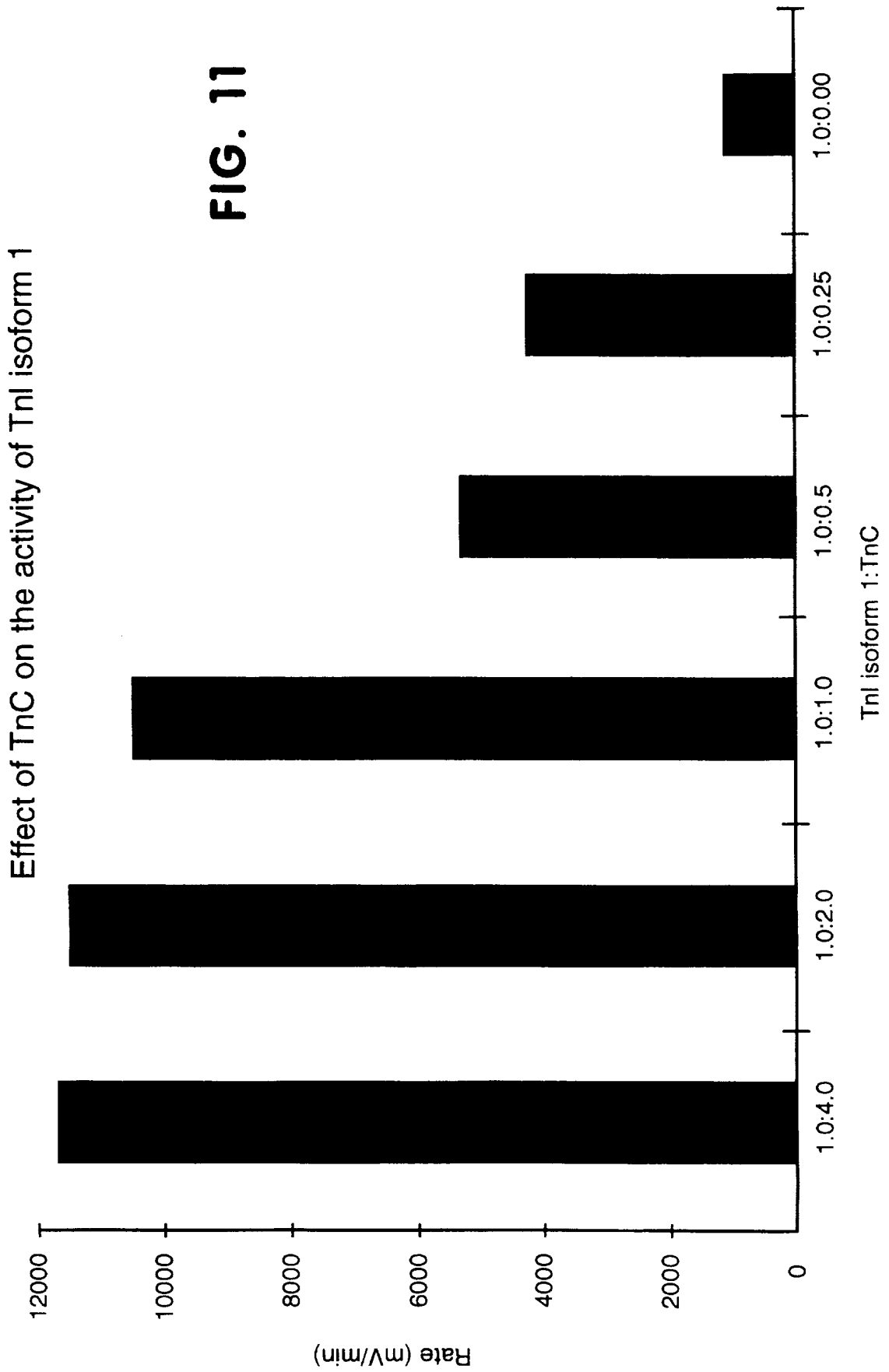
		1:1	2:1	3:1	1:1	2:1	3:1
	TnC	r-Tnl•TnC		Tnl isoform•TnC			
Lane	1	2	3	4	5	6	7

**FIG. 9**

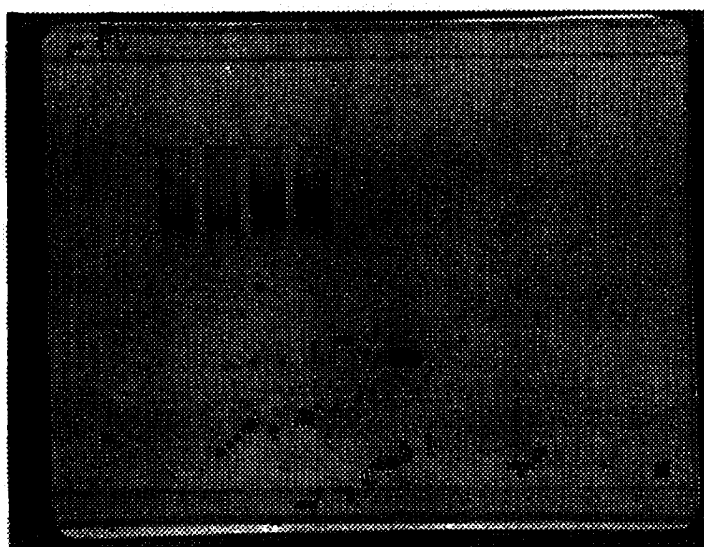
FIG. 10



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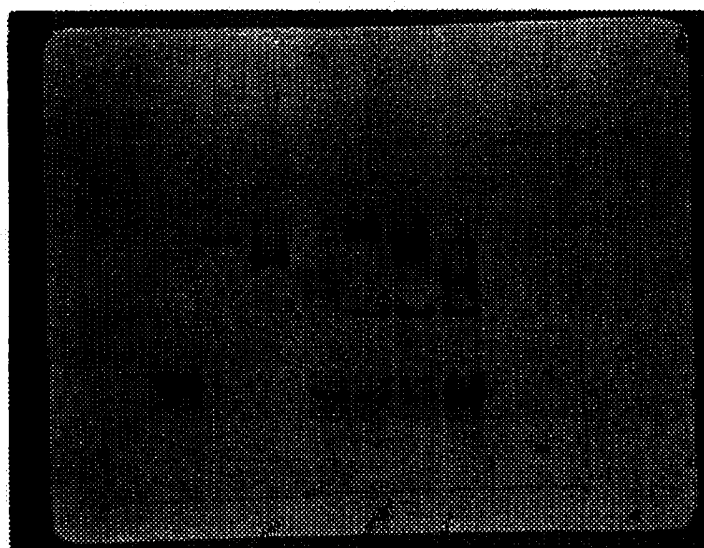
# Polyacrylamide Gel Electrophoresis of TnI isoform I:TnC:TnT complex.



1 2 3 4 5 6 7

**FIG. 12**

# Polyacrylamide Gel Electrophoresis of TnI Isoform II:TnC Complex.

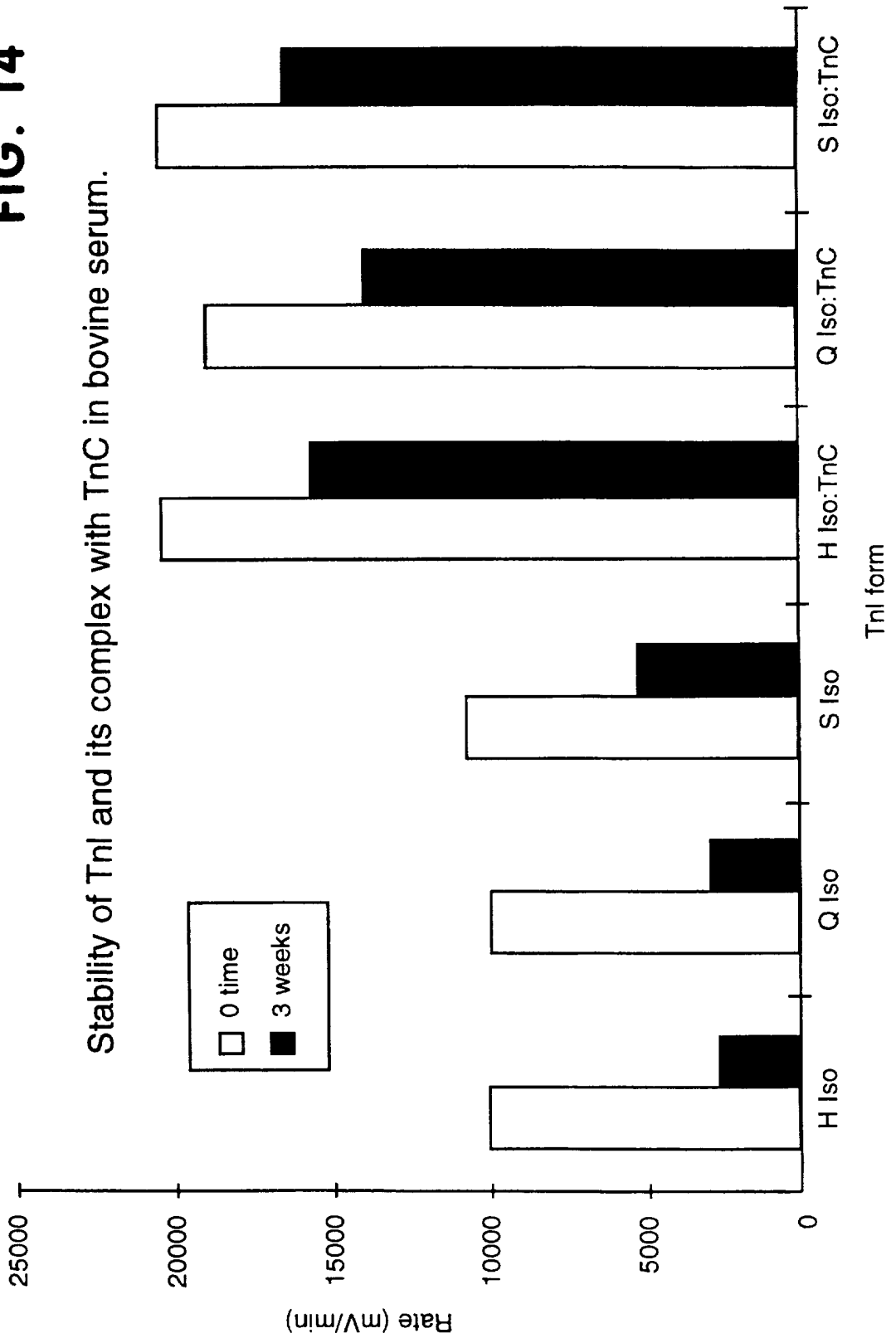


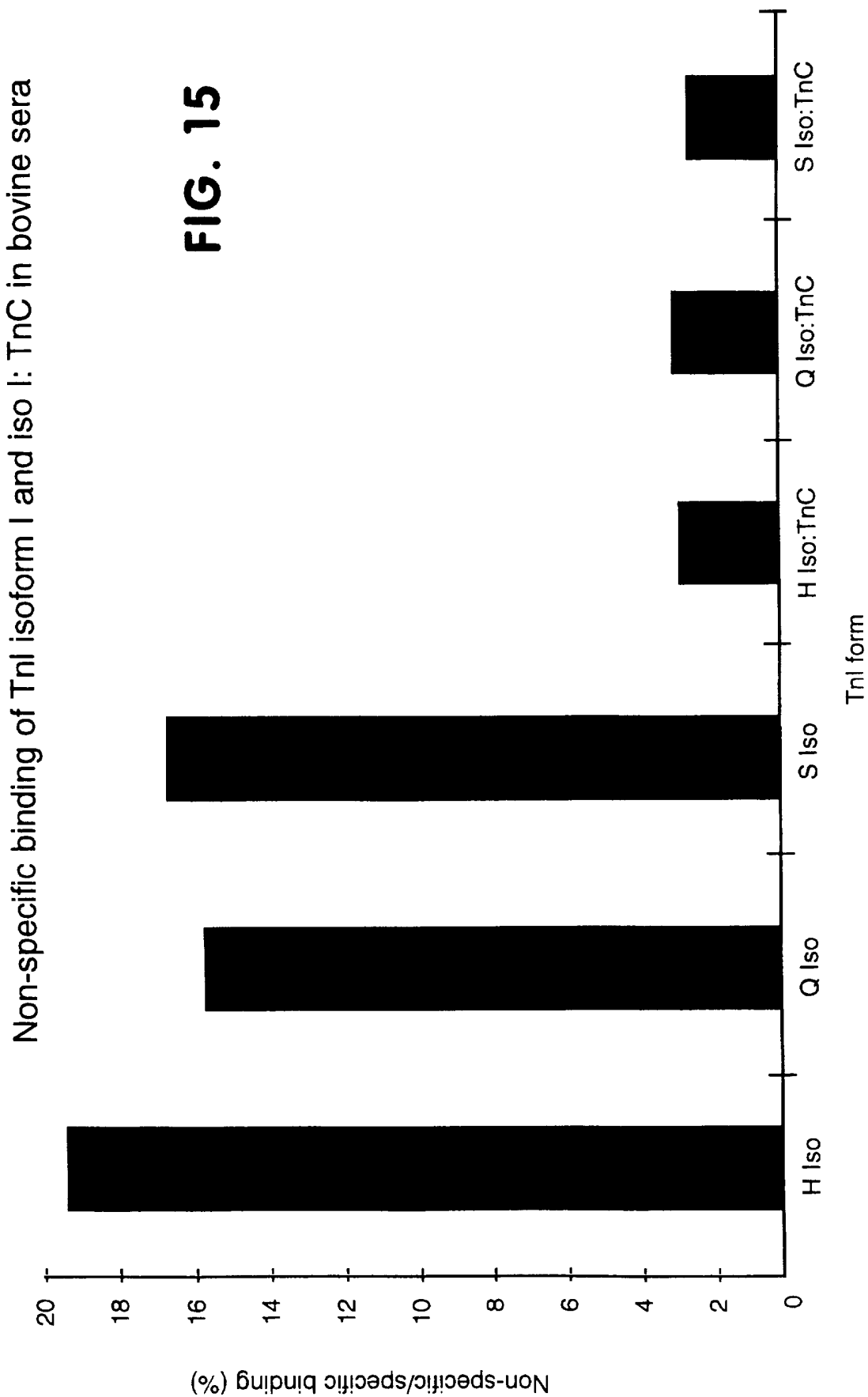
1 2 3 4 5 6 7

**FIG. 13**

**FIG. 14**

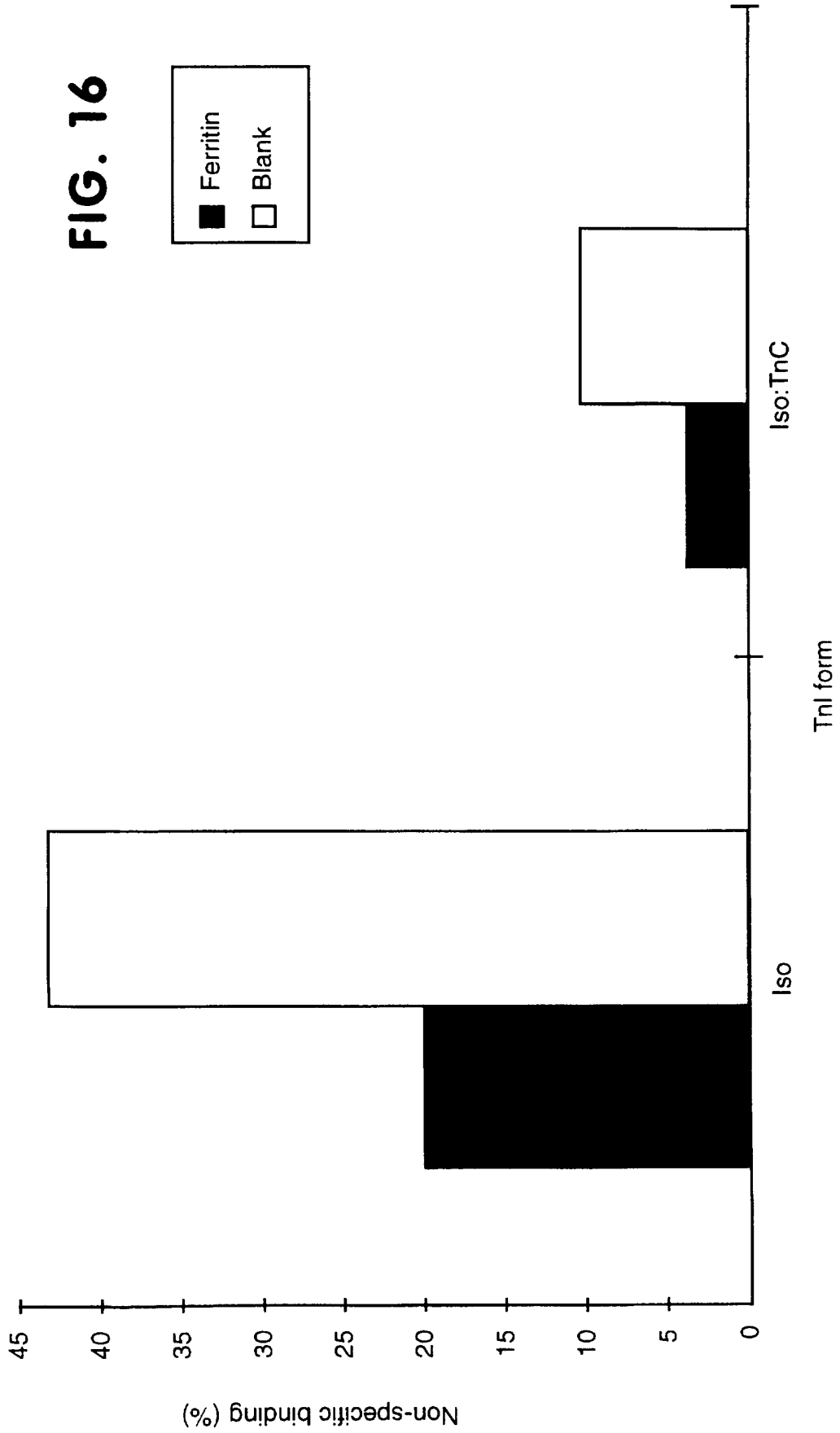
Stability of Tnl and its complex with TnC in bovine serum.





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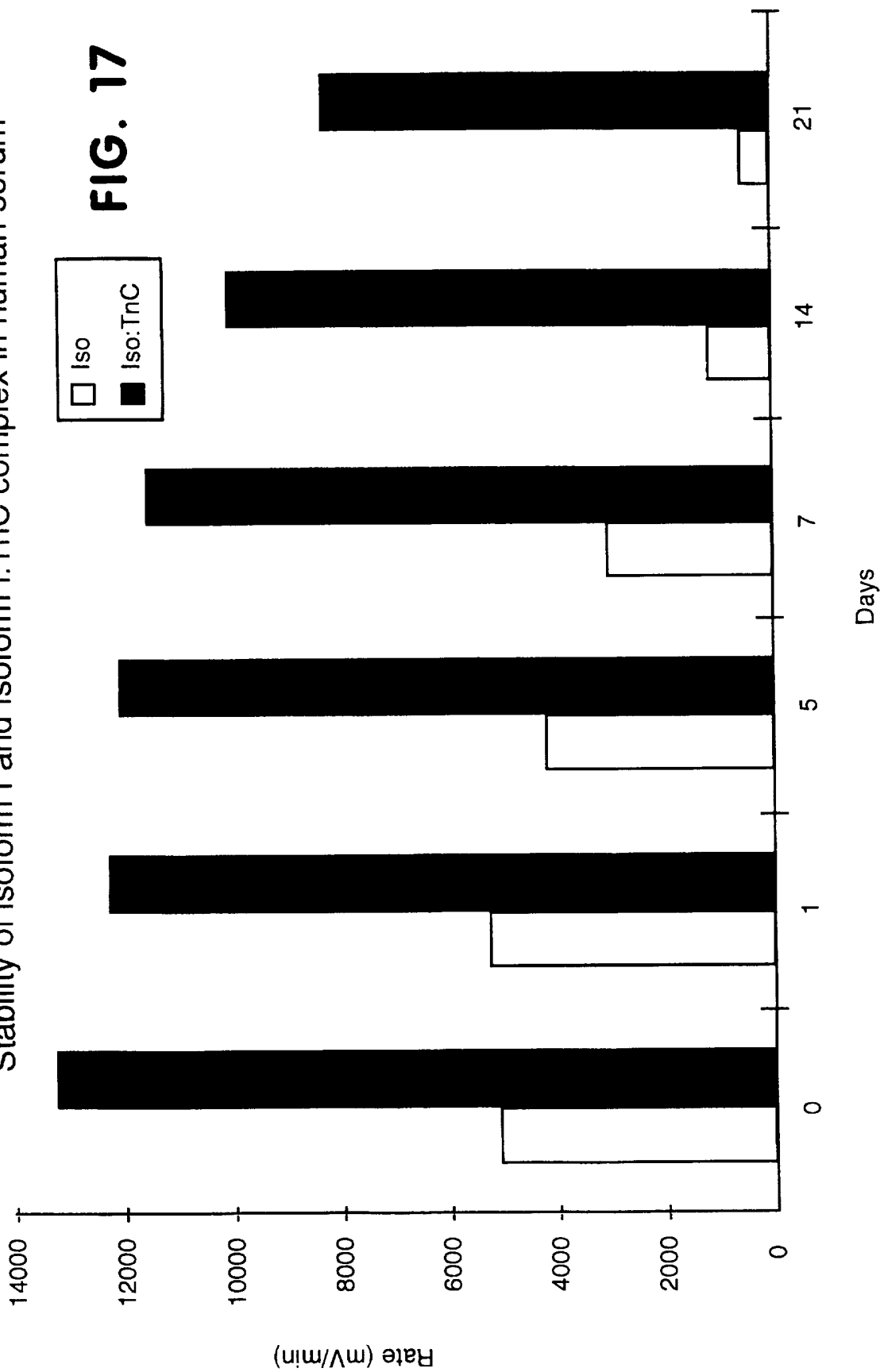
Non-specific binding of Tnl isoform and iso: TnC in human sera



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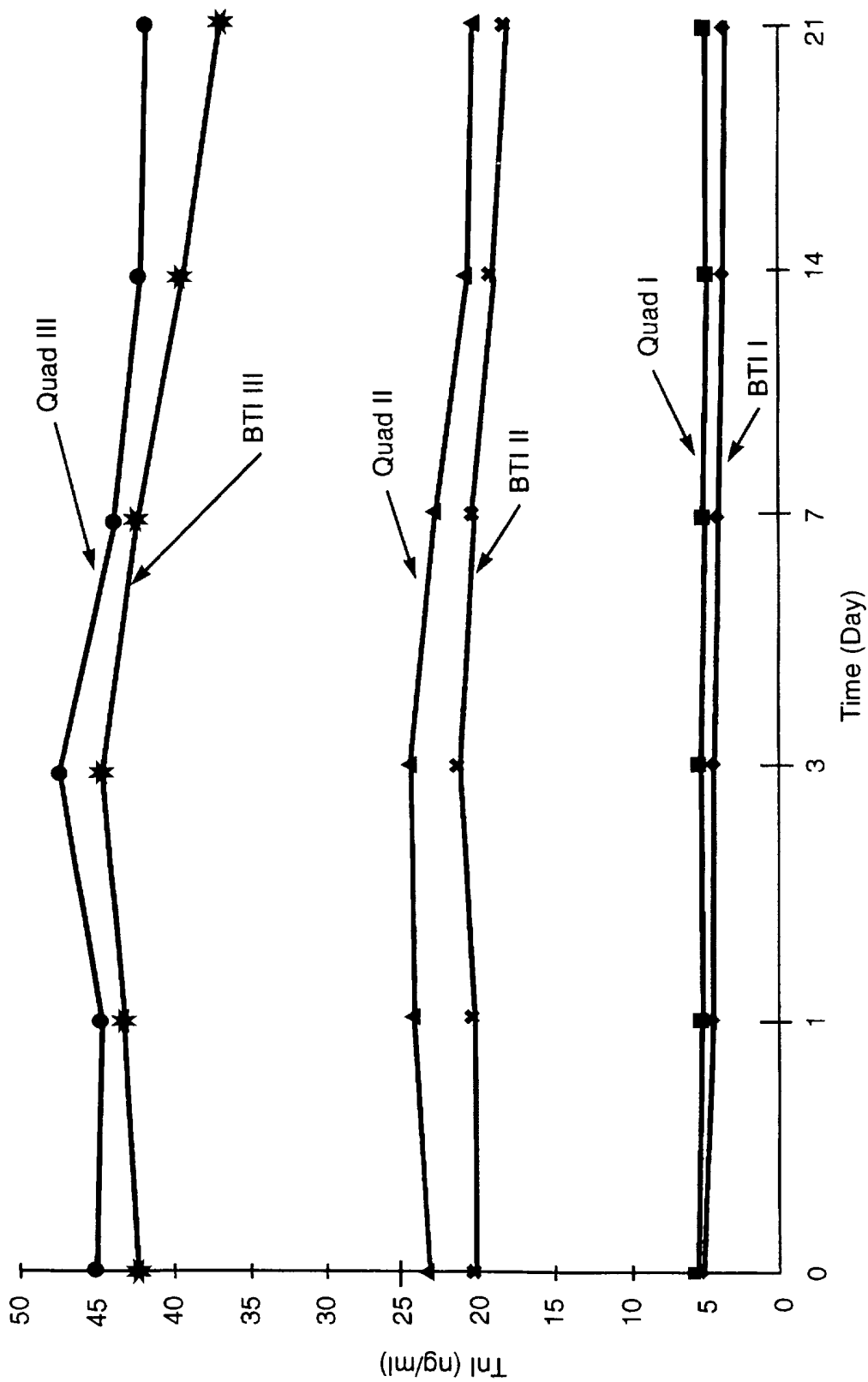
Stability of isoform I and Isoform I:TnC complex in human serum

**FIG. 17**

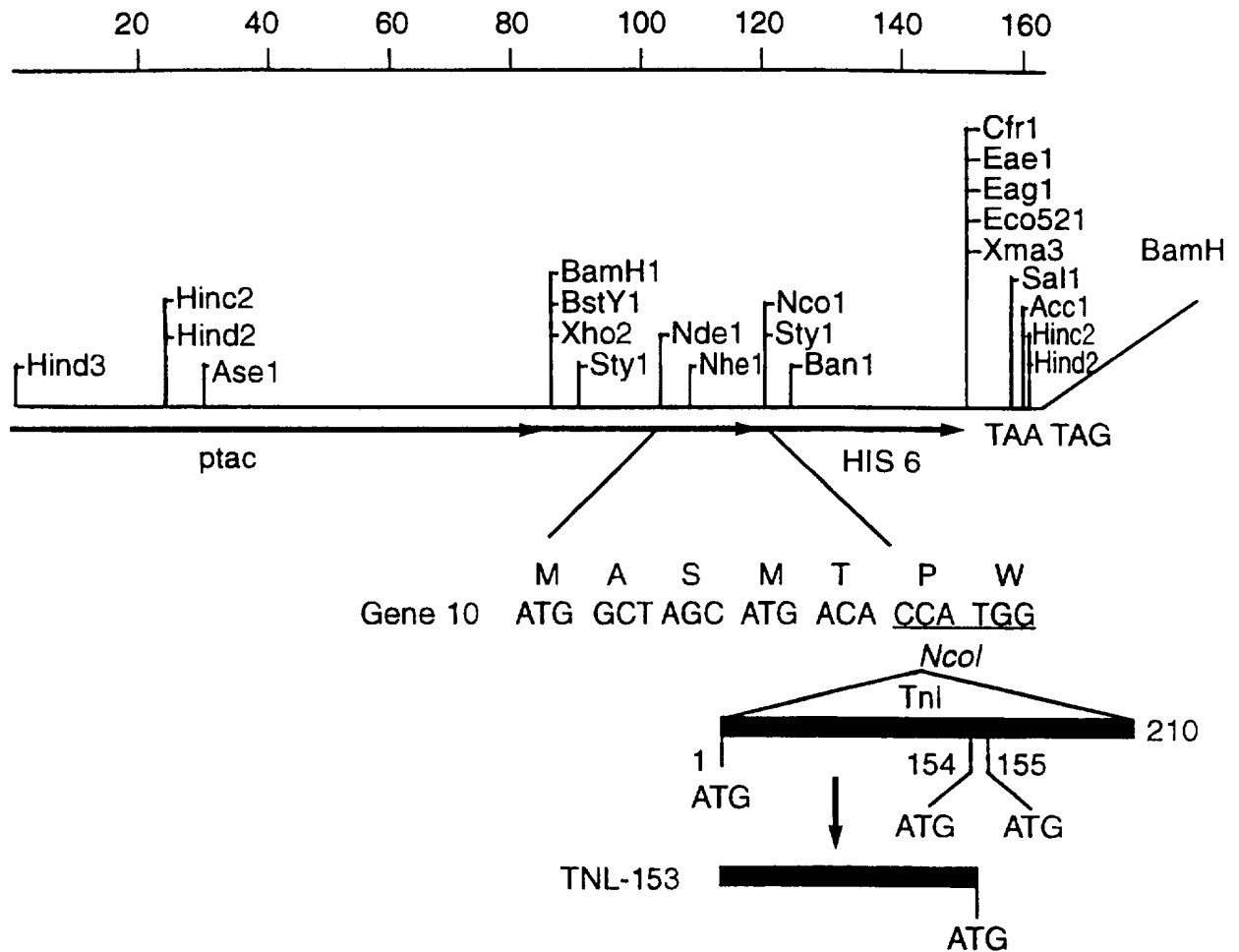


**FIG. 18**

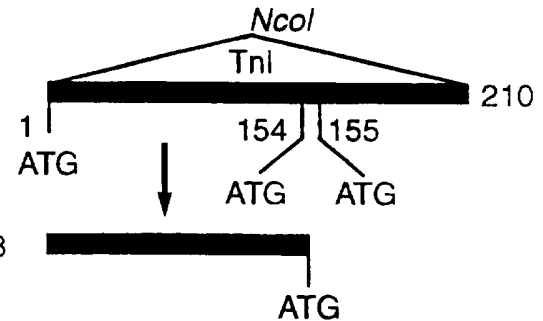
Reconstituted Stability of TnI Isoform I:TnC in Bovine Sera



**FIG. 19** Map of expression Vector  $p^{tac}$ /Gene10/Troponin I/6xHis



Gene 10 M A S M T P W  
 ATG GCT AGC ATG ACA CCA TGG



Tac Promotor

Hind III \_\_\_\_\_

AAGCTTACTCCCCATCCCCCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATA  
 TTCGAATGAGGGGTAGGGGGACAACCTGTTAATTAGTAGCCGAGCATATTACACACCTTAACACTCGCCTAT

\_\_\_\_\_ BamHI \_\_\_\_\_ Gene 10 RBS \_\_\_\_\_ Nco I \_\_\_\_\_ 6x His tag

ACAATTTACACAGGATCCAAGGAGATATACATATGGCTAGCATGACACCATGGCCACCATCACCACCAT  
 TGTTAAAGTGTGTCCTAGGTTCTCTATATGTATACCGATCGTACTGTGGTACCGGGTGGTAGTGGTGGTA

\_\_\_ Stop Not I Sal I

CACTAATAGGCGGCCGCGTCGAC  
 GTGATTATCCGCGGCGCAGCTG

CCATGG - (1st aa . . coding sequence for expression . . last aa) - N - CCATGG

# INTERNATIONAL SEARCH REPORT

Internat. Application No.  
PCT/US 96/18878

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/47 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FASEB JOURNAL FOR EXPERIMENTAL BIOLOGY, vol. 9, no. 6, 24 April 1995, BETHESDA, MD US, page A-1469, abstract no. 1232 XP002028677 N MORJANA ET AL.: "The reversible denaturation of cardiac-troponin-I" see the whole document ---	7,8
X	BIOCHEMISTRY, vol. 27, no. 8, 19 April 1988, EASTON, PA US, pages 2821-2827, XP002028678 J LESZSK ET AL.: "Amino acid sequence of bovine cardiac troponin I" see the whole document --- -/--	7,8

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- \*&\* document member of the same patent family

Date of the actual completion of the international search

2 April 1997

Date of mailing of the international search report

11.04.97

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Authorized officer

Masturzo, P

# INTERNATIONAL SEARCH REPORT

Internatic Application No PCT/US 96/18878
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JOURNAL OF BIOCHEMISTRY, vol. 117, no. 1, January 1995, TOKYO JP, pages 158-162, XP002028679 T OJIMA ET AL.: "Amino acid sequence of C-terminal 17kDa CNBr fragment of Akazara scallop troponin-I" see the whole document ---</p>	1-11
P,X	<p>FASEB JOURNAL FOR EXPERIMENTAL BIOLOGY, vol. 10, no. 6, 30 April 1996, BETHESDA, MD US, page A-1295, abstract 1706 XP002028680 N A MORJANA &amp; C H DEMARCO: "Biochemical and immunological properties of a cyanogen bromide fragment of human cardiac troponin I" see the whole document -----</p>	1-11