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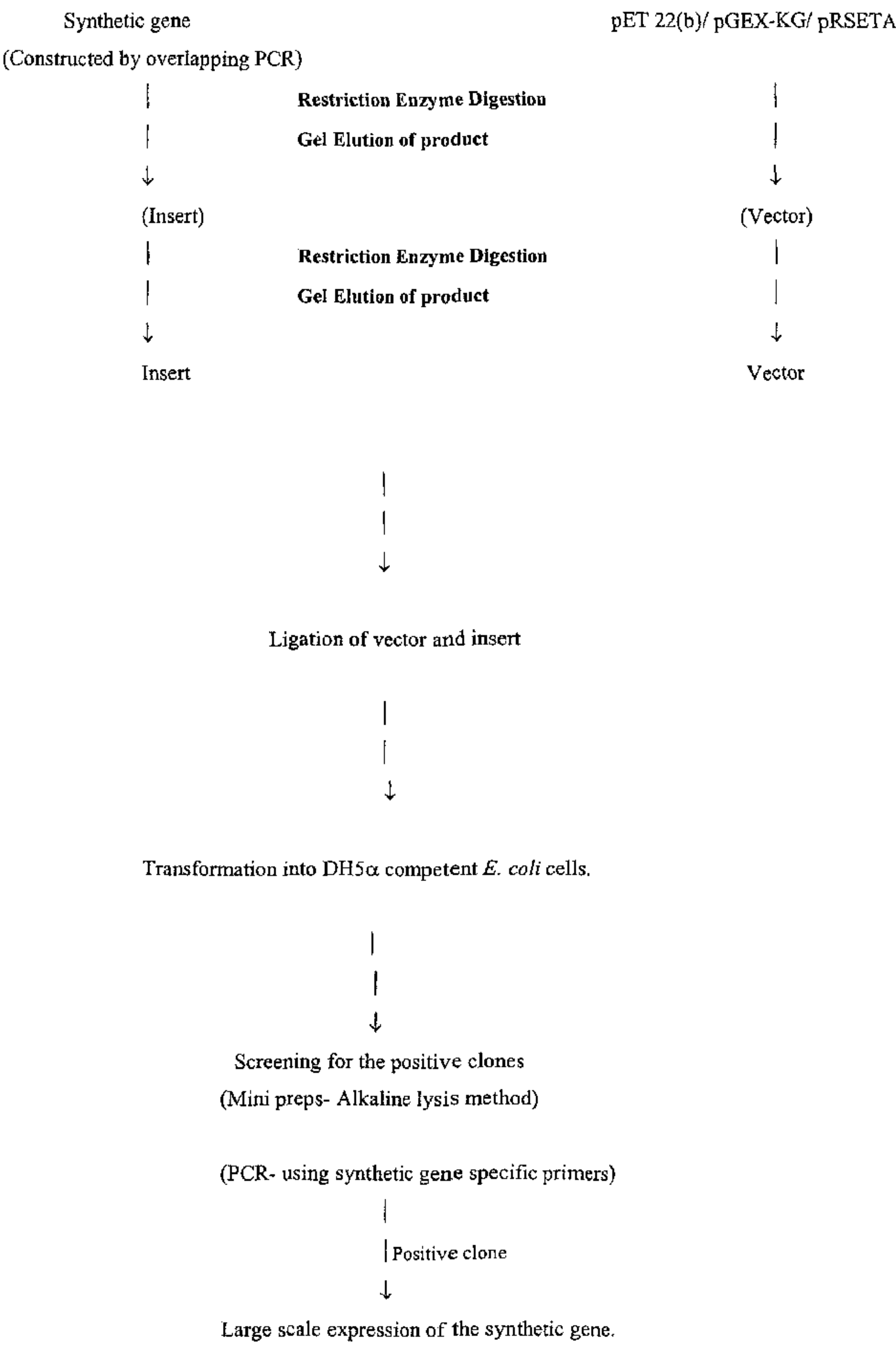
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(54) Title: MULTIVALENT SYNTHETIC VACCINE FOR CANCER



(57) Abstract: Multivalent vaccine comprising peptides from vasoactive intestinal peptide, bombesin, Substance P and epidermal growth factor are described. A method of constructing a multivalent gene for use in various expressions vectors and the protein recombinantly expressed in the prokaryotic expression systems are also described.

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MULTIVALENT SYNTHETIC VACCINE FOR CANCER**Field Of The Invention**

The present invention is in the field of molecular biology, immunology and medicine, and it relates to a synthetically prepared multivalent vaccine for cancer.

5 The vaccine of this invention comprises of a polypeptide which is composed of Vasoactive intestinal peptide (VIP), Bombesin, Substance P and Epidermal growth factor (EGF). The vaccine of this invention comprises the peptides expressing VIP, SP, Bombesin and EGF. The present invention relates to the design and construction of a synthetic polypeptide coupled through linkers. The present invention also relates to the construction of the synthetic gene to the respective designed polypeptide. It also relates to the synthesis of the multivalent gene by Splice-Overlap-Extension PCR. The constructed multivalent gene is then used for cloning in various expression vectors and the protein recombinantly expressed in the prokaryotic expression system. In the present invention the recombinantly expressed protein was also used to study its immunological response in the murine model.

The invention will be useful as a pharmaceutical composition for the prevention and treatment of various forms of cancers. More particularly, the present work relates to the preparation of human anti- cancer vaccines useful for the prevention and treatment of cancers such as colon, rectum, lung, breast, brain, pancreas, prostate, liver, gastrointestinal, thyroid, ovary, head and neck, kidney, melanomas, neuro-blastoma, glioblastoma leukemia and lymphomas in mammals, particularly humans.

**Description Of The Prior Art**

Cancer vaccines are on the threshold of taking their place alongside the more traditional cancer treatment modalities of surgery, radiation therapy and chemotherapy (*Curr Opin Mol Ther*, 3(1): 77- 84, 2001). The treatment of cancer requires increased tumor protective immunity by active immunization to tumor antigens. The epitope- based approach is clearly compelling in the approach to vaccine strategy. A relatively tiny, but immunologically relevant sequence is often capable of inducing protective immunity against large and complex pathogen. (*Immunol. And Cell. Biol*; 75:402- 408, 1997). Scott, A.T., *et al.* have reported the construction of a recombinant vaccinia virus coding for an artificial polyepitope protein containing 10 contiguous minimal CTL epitopes which were restricted by five MHC alleles and derived from five



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viruses, a parasite and a tumor model. The construct coding for this protein was capable of inducing MHC- restricted primary CTL responses to all 10 epitopes (*J. Immunol*, 1196, 157:822-826).

Recent reports reflect that a multi-antigen vaccine for cancer would be  
5 advantageous. Firstly, by simultaneously attacking multiple antigen targets, such a vaccine might limit immune evasion by tumors, which have reduced expression of individual target antigen. Secondly, such a vaccine might prime for protective CTL responses directed towards antigens not previously recognized by the immune system.

A common theme in human cancers is the stimulation of tumor cell  
10 growth by autocrine and paracrine signaling .In addition to polypeptide growth factors such as EGF, recent evidences supports the role of specific neuropeptides in gastric, colorectal, pancreatic and prostatic cancers. (*Oncogene* 2001, Mar 26;20(13):1563-9). These neuropeptides have defined physiological functions such as neurotransmitters.

Vasoactive intestinal peptide (VIP) is a 28 amino acid neuroendocrine  
15 mediator with a broad range of biological activities in diverse cells and tissues. VIP receptors are expressed on adenocarcinomas, breast cancers, melanomas and pancreatic carcinomas. (*Cancer Res.*; 54, 690- 700,1994). Elements within the entire primary sequence are necessary for recognition by the receptors. Today VIP is widely considered to have a physiological regulatory influence on a wide spectrum of body  
20 functions, being involved in the pathogenesis of several human disorders and offers hope for the improved prevention or treatment of certain diseases (*Immunology Today*; 21, No 1; 2000). In children suffering from neuroblastoma, VIP may stimulate or inhibit tumor growth, in an autocrine fashion (*Adv. Neuroimmunol*, 6: 29-36, 1996). VIP has been shown to possess growth factor effects on mammary cancer, prevalent in adult  
25 female population. (*Cancer* 67: 1561-1564, 1991). VIP1 receptors are present in breast cancer cells. VIP hybrid is a VIP receptor antagonist that inhibits breast cancer proliferation. A VIP analog has been developed for imaging breast tumors. VIP 1 receptors may be utilized for the early detection and treatment of breast cancers (*Ann NY Acad. Sci* 11, 865: 290-296, 1998). VIP also exhibits differential effects on the invasive  
30 potential, rapid progression, and aggressive clinical course of prostate cancer, via the cAMP production (*J. Urol.* 149:1209-1213; 1993). In many lung cancer cell lines, VIP stimulates mitosis, and the hybrid VIP antagonist inhibits the VIP- stimulated mitosis,



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both *invitro* and *in vivo*, suggesting a possible suggesting role for VIP antagonists in lung cancer inhibition (*Biomed Res. 13: 131-135, 1992*).

Neuropeptides such as Substance P (SP) and Bombesin regulate many biological processes through binding to and activating their respective cell surface  
5 receptors. Many astrocyte/ glial- derived brain tumor cell lines express functional SP and bombesin receptors. Activation of these neuropeptide receptors stimulates several signaling pathways that regulate transcription and translation leading to the induction of mitogenesis in several cell types including astrocyte brain tumor -derived cell lines (*Int. J. Oncol, 12(2): 273- 86 1998*).

10 Bombesin is a tetradecapeptide which typically act as neurotransmitters along the brain- gut axis and as growth factors in various human tissues (*J. Am. Soc. Nephrol 2000, 11(8): 1409- 1418*). Cancers of the GI tract, breast, lung and prostate frequently express the peptide and its receptors. Most studies have found that bombesin acts to increase tumor cell proliferation, leading to the hypothesis that this peptide  
15 hormone is a mitogen important for growth of various cancers (*Peptides; 2001: 22(4): 689-99*). Malignant transformation of human kidney tissue into renal cell carcinoma is also accompanied by novel expression of the bombesin receptors. Antagonists to bombesin have been proposed as agents for the treatment of cancers. U.S.A. Patent No. U.S. 6,083,915 refers to a method of treating hepatoma, or liver cancer, in mammalian  
20 subjects by administering them with a composition containing a therapeutically effective amount of the bombesin analog. U.S.A. Patent No U.S. 5,217,955 refers to a method of treating cancer in a human patient. The method involves administering to the patient analog of a naturally occurring biologically active peptide of mammalian gastrin-releasing peptide, neuromedin B, neuromedin C, bombesin, or litorin. The combination  
25 could be used for the treatment of colon, prostate and breast cancer.

Substance P (SP) is a neuropeptide found in both the central and peripheral nervous system. SP is expressed in tumors arising from neural crest derived cells including neuroblastomas, neuro-endocrine carcinomas, medullary carcinomas of the thyroid, etc (*J. Cutan Pathol; 25, 2-10, 1998*). SP derivatives are potential  
30 therapeutic compounds for the treatment of SCLC. [Arg<sup>6</sup>, D-Trp<sup>7,9</sup>, N<sup>me</sup>Phe<sup>8</sup>]- substanceP (6-11) (antagonist G), a derivative of SP is a novel anti- cancer agent which is entering phase II clinical trials for the treatment of SCLC. (*Br. J. cancer 83, 941-948, 2000*). SP



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also have a pro- angiogenic function, stimulates endothelial cell differentiation into capillary- like structures in a dose dependent manner. (*Euro. J. Pharmacol* 18, 298: 335-338).

EGF is a single polypeptide of 53 amino acid residues, which is involved  
5 in the regulation of cell proliferation and mitogenesis. The peptide appears to be non-glycosylated and is very stable (*Cell Biology International, Vol. 19, 5, 1996*). The biological effect of EGF is mediated by binding of EGF with its specific cell- surface receptors and culminates in biochemical events that lead to DNA synthesis (*J. of Cellular Physiol*; 148: 220- 227, 1991). There is evidence of a relationship between  
10 EGF and tumor cell proliferation, such as the overexpression of EGF receptor (EGFR) in different human tumors, which makes this system an interesting target for cancer treatment (*Annal. Oncol* 9,431-5, 1998). In a study carried out in Philadelphia, USA, a naturally occurring mutant human EGF receptor (EGFRvIII) was tried as a target for peptide vaccine immunotherapy of tumors (*Cancer Res. 15; 57(8); 1419-24, 1997*).  
15 This spontaneously occurring alteration is found in a high percentage of primary human brain, breast, lung and ovarian tumors. Pre immunization of mice with this peptide substantially increased tumor formation of cells expressing EGFRvIII. Thus, the alteration present in EGFRvIII could serve as the basis for an anti-tumor vaccine with potentially wide applications in humans. Growth inhibitory action of EGF has been  
20 reported in epidermal carcinoma A431 cells, which have an unusually high density of cell surface EGF receptors (*Cell Growth Differ* 7(2): 173-8, 1996). U.S.A. Patent Application Number 5,894,018, refers to a vaccine composition comprising autologous EGF coupled to a carrier protein such as tetanus toxoid or cholera toxin B chain. An important objective of this invention was to obtain a vaccine composition for the active  
25 immunotherapy of EGF dependent malignant tumors, for, e.g. Epidermal carcinoma of lung, glioblastoma multiforme and head and neck epidermoid carcinoma.

U.S. Patent No. 6,156,725, refers to the use of a combination of peptides, viz, Somatostatin, bombesin, VIP and substance P to block the uncontrolled multiplication of cancer cells of the colon, rectum, lung, breast, and kidney. The  
30 invention also relates to a pharmaceutical composition containing a combination of such analogs. The combination may also be useful in preventing, inhibiting, or modulating the hypersecretion of VIP, bombesin, substance P or a combination of all.

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## SUMMARY OF THE INVENTION

The present invention describes the design of the polypeptide consisting of the four peptides viz. Vasoactive Intestinal Peptide (VIP) (SEQ ID NO:1), Bombesin (Bom) (SEQ ID NO:2), Substance P (SP) (SEQ ID NO:3) and Epidermal Growth Factor (EGF) (SEQ ID NO:4); which are covalently linked with or without linkers and may be useful as vaccine for the treatment of cancer. The preferred designed polypeptide sequence has a total of 112 aminoacids as described in (SEQ ID NO:5) in which the four mentioned peptides are linked through Gly-Gly linker. The present invention also relates to the design of corresponding synthetic gene using codon optimization for the prokaryotic system and the sequence of the designed synthetic gene is described in SEQ ID NO:6. It also describes the method for the construction of complete multivalent synthetic gene, its cloning in various prokaryotic vectors and expression in the *E.coli* system. The method of purification and characterization of the expressed recombinant protein has also been described in this patent application. It also describes production of polyclonal antibodies and T-cell immune response in the murine model, using the recombinantly expressed protein.

The recombinant protein has been synthesized with the presumption that a multi-epitope vaccine could induce immunity against multi antigenic targets and hence could be used for the treatment of a broad spectrum of cancers. This present invention could prove helpful in preventing, inhibiting, or modulating the hypersecretion of VIP, bombesin, substance P and EGF or a combination of all.

## BRIEF DESCRIPTION OF FIGURES

Fig. 1: shows the schematic depiction of the PCR method to be followed in the construction of the synthetic multivalent vaccine using the oligonucleotides depicted in SEQ ID NO:7-14.

Fig. 2: schematically depicts the strategy to be followed for the cloning and expression of the constructed gene in appropriate vectors.

Fig. 3: SOE in PCR of Multivalent gene. After each PCR, the product was gel eluted (using Qiagen Kit) and used as the template for the next - step of PCR. Lane1; 100bp ladder; Lane2: 126 bp DNA (after first step- PCR); Lane 3: 179 bp DNA (after second- step PCR); Lane 4: 232 bp DNA (after third- step PCR); Lane 5: 285 bp

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DNA (after fourth- step PCR); Lane 6: 336 bp DNA (after fifth step PCR); Lane 7: 336bp DNA (after final step PCR); Lane 8: 100 bp ladder.

Fig. 4: shows the vector map of pET- 22b(+).

Fig. 5: shows the vector map of pGEX 5X2.

5 Fig. 6: shows the vector map of pPRSET A.

Fig. 7: Cloning in pET-22b(+)- Screening of positive clones by restriction enzyme digestion. Plasmid DNA isolation of the clones was done by Alkaline SDS Method. For identifying the positive clones, plasmid DNA was digested with the restriction enzymes EcoR1 and Not 1. The digested sample was run on a 1.8% analytical agarose gel. Lane 1- 6: mini prep samples; Lane 7: 100bp ladder.

Fig. 8: depicts the nucleotide sequence of clone #2 pET- MV (SEQ ID NO:18). Fig. 8 (SEQ ID NO:18) depicts the nucleotide sequence of the clone pET MV#2. The highlighted nucleotides e.g. “g” and “a” (in bold, underlined and italicized) show the substitutions that have taken place in this clone, as compared to the original designed multivalent nucleotide sequence as depicted in SEQ ID NO:6

Fig. 9: depicts the amino sequence of clone #2 pET- MV (SEQ ID NO:19). Fig. 9 (SEQ ID NO:19) depicts the aminoacid sequence of the clone pET MV#2, which has been extrapolated from the corresponding nucleotide sequence (Fig. 8; SEQ ID NO:18), using the translational tool software. The highlighted aminoacid e.g. “Gly” and “Ser” (in bold, underlined and italicized) show the substitutions that have taken place in this clone, as compared to the original designed multivalent aminoacid sequence as depicted in SEQ ID NO:5.

Fig. 10: Cloning in pGEX5X-2- Screening of positive clones by restriction enzyme digestion. Plasmid DNA isolation of the clones was done by Alkaline SDS Method. For identifying the positive clones, plasmid DNA was digested with the restriction enzymes EcoR1 and Not 1. The digested sample was run on a 1.8% analytical agarose gel. Lane 1- 7: mini prep samples; Lane 8: 100bp ladder.

Fig. 11: depicts the nucleotide sequence of clone #16 pGEX- MV (SEQ ID NO:20). Fig. 11 (SEQ ID NO:20) depicts the nucleotide sequence of the clone pGEX MV#16. The clone shows 98% homology with the original designed multivalent nucleotide sequence (SEQ ID NO:6). The reading frame of the clone has been maintained till the 95<sup>th</sup> nucleotide codon. At the 95<sup>th</sup> codon, there is an addition of a



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nucleotide “t”, followed by three substitutions viz., “a”, “g” and “t” (in bold, underlined and italicized). Consequently, there has been a reading frame- shift, leading to an inclusion of a stop codon viz. “tga” at the 99<sup>th</sup> codon position.

Fig. 12: depicts the amino sequence of clone#16 pGEX – MV (SEQ ID NO:21). Fig. 12 (SEQ ID NO:21) depicts the aminoacid sequence of the clone pGEX MV # 16, which has been extrapolated from the corresponding nucleotide sequence (Fig. 11; SEQ ID NO:20), using the translational tool software. The changes in the aminoacid sequence have been highlighted. Due to the incorporation of a stop codon, the last 14 amino acids are not expressed. Also, the 3 amino acids before the stop codon have been altered due to the substitutions and additions at the nucleotide level.

Fig. 13: Cloning in pET-22b(+)[incorporating a stop codon]- Screening of positive clones by restriction enzyme digestion. Plasmid DNA isolation of the clones was done by Alkaline SDS Method. For identifying the positive clones, plasmid DNA was digested with the restriction enzymes EcoR1 and Not 1. The digested sample was run on a 1.8% analytical agarose gel. Lane 1- 7: mini prep samples; Lane 8: 100bp ladder.

Fig. 14: depicts the nucleotide sequence of clone# 1 pET- MVSC (SEQ ID NO:22). Fig. 14 (SEQ ID NO:22) depicts the nucleotide sequence of the clone pET MVSC#1. The highlighted nucleotide e.g. “g” (in bold, underlined and italicized) show the substitution that has taken place in this clone, as compared to the original designed multivalent nucleotide sequence as depicted in SEQ ID NO:6. Since the substitution was at the 3<sup>rd</sup> position in the codon, the amino acid coded by the codon remained.

Fig. 15: depicts the amino acid sequence of clone#1 pET- MVSC (SEQ ID NO:23). Fig. 15 (SEQ ID NO:23) depicts the aminoacid sequence of the clone pET MV SC # 1, which has been extrapolated from the corresponding nucleotide sequence (Fig. 14; SEQ ID NO:22), using the translational tool software. The aminoacid sequence shows 100% homology to the original designed multivalent aminoacid sequence as depicted in SEQ ID NO:5.

Fig. 16: depicts the nucleotide sequence of clone # 9 pET- MVSC (SEQ ID NO:24). Fig. 16 (SEQ ID NO:24) depicts the nucleotide sequence of the clone pET MVSC #9. The clone showed 100% homology with the original designed multivalent nucleotide sequence as depicted in SEQ ID NO:6

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Fig. 17: depicts the amino acid sequence of clone #9 pET- MVSC (SEQ ID NO:25). Fig. 17 (SEQ ID NO:25) depicts the aminoacid sequence of the clone pET MV SC # 9, which has been extrapolated from the corresponding nucleotide sequence (Fig. 16, SEQ ID NO:24), using the translational tool software. The aminoacid sequence shows 100% homology to the original designed multivalent aminoacid sequence as depicted in SEQ ID NO:5.

Fig. 18: depicts the nucleotide sequence of clone # 14 pET- MVSC (SEQ ID NO:26). Fig. 18 (SEQ ID NO:26) depicts the nucleotide sequence of the clone pET MVSC # 14. The highlighted nucleotides e.g. “t” (in bold, underlined and italicized) shows the substitution that has taken place in this clone, as compared to the original designed multivalent nucleotide sequence as depicted in SEQ ID NO:6

Fig. 19: depicts the amino acid sequence of clone #14 pET- MVSC (SEQ ID NO:27). Fig. 19 (SEQ ID NO:27) depicts the aminoacid sequence of the clone pET MV SC # 14, which has been extrapolated from the corresponding nucleotide sequence (Fig. 18, SEQ ID NO:26), using the translation tool software. The highlighted aminoacid e.g. “Glu” (in bold, underlined and italicized) shows the substitution that has taken place in this clone, as compared to the original designed multivalent aminoacid sequence as depicted in SEQ ID NO:5

Fig. 20: SDS- PAGE profile of expression of pET- MV2 at 30°C: pET- MV2 was expressed in BL21DE3λ. The culture was induced with 1mM IPTG at 30°C and expression of the protein was studied at various time points. At each time point, the induced and uninduced aliquots (1-ml) of the culture were pelleted down. The pellet was boiled in 5X SDS- PAGE loading dye, boiled for 10 minutes and 15 ul of the sample was loaded on a 15% SDS- PAGE. Lane 1: Induced sample post 2 hour of IPTG addition; Lane 2: Uninduced sample post 2 hour; Lane 3: Induced sample post 2<sub>1/2</sub> hour of IPTG addition; Lane 4: Uninduced sample post 2<sub>1/2</sub> hour.

Fig. 21: SDS- PAGE profile of expression of pET- MV2 at 37°C: pET- MV2 was expressed in BL21DE3λ. The culture was induced with 1mM IPTG at 37°C and expression of the protein was studied at various time points. At each time point, the induced and uninduced aliquots (1-ml) of the culture were pelleted down. The pellet was boiled in 5X SDS- PAGE loading dye, boiled for 10 minutes and 15 ul of the sample was loaded on a 15% SDS- PAGE. Lane 1: Uninduced sample post 2 hour of



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IPTG addition; Lane 2: Induced sample post 2 hour; Lane 3: UnInduced sample post 2<sub>1/2</sub> hour of IPTG addition; Lane 4: Induced sample post 2<sub>1/2</sub> hour. Lane 5: molecular weight marker.

Fig. 22: Immunoblot of pET- MV2: pET-MV2 was expressed in BL21DE3λ. The culture was induced with 1mM IPTG at 30°C. After 2<sub>1/2</sub> hour of IPTG induction, cells were pelleted down. The pellet was boiled in 5X SDS- PAGE loading dye, boiled for 10 minutes and 15 µl of the sample was loaded on a 15% SDS- PAGE. The proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA and probed with anti- peptide antibodies. Lane 1: Immunoblot of induced crude extract with anti- VIP antibody; Lane 2: Immunoblot of crude extract with anti- His antibody; Lane 3: Immunoblot of induced crude extract with anti- SP antibody; Lane 4: Immunoblot of induced crude extract with anti- EGF; Lane 5: Immunoblot of uninduced crude extract with anti- SP antibody.

Fig. 23: Immunoblot of purified multivalent protein: The crude extract of the induced pET- MV2 clone was loaded on a Ni- NTA column. The protein was eluted with increased concentration of imidazole. The protein samples were loaded on 15% SDS- PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA and probed with anti- SP antibody. Lane 1: Crude extract; Lane 2: Purified fraction; Lane 3: Purified fraction.

Fig. 24: SDS- PAGE of gel purified multivalent protein: The crude extract of the induced pET- MV2 clone was loaded on 15% SDS- PAGE. Electrophoresis was done at constant voltage of 100V for about 3hrs. As described in the example, the portion of the gel corresponding to the desired protein was cut, washed with 2mM Phosphate buffer (pH 6.8) minced and put in a dialysis tubing (Mol wt. Cut off- 3,500Da). Electroelution was carried out at 40 V (for 40 min at 4°C) in a buffer tank containing 2mM Phosphate buffer (pH 6.8). The protein sample was taken out from the dialysis bag and was concentrated in a speed vac, under cold conditions. It was reconstituted in minimal volume of 2mM PBS and loaded on a 15% SDS-PAGE gel. Lane1: Mol wt marker, Lane3: Eluted protein; Lane 4: Crude extract.

Fig. 25: Immunoblot of gel purified multivalent protein: The electroeluted protein was loaded on a 15% SDS-PAGE gel. The proteins were then electrophoretically transferred to a nitrocellulose membrane, blocked with 3% BSA and



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probed with anti- peptide antibodies. Fig. 25 A. Immunoblot with anti- SP antibody  
Lane 1: Crude extract; Lane 2: Electroeluted protein Fig. 25. B. Immunoblot with anti-  
VIP antibody Lane 1: Crude extract; Lane2:Electroeluted protein.

Fig. 26: SDS- PAGE profile of expression of pGEX- MV16 at 30°C:

5 pGEX MV16 was expressed in BL21DE3λ. The culture was induced with 1mM IPTG  
at 30°C and expression of the protein was studied at various time points. At each time  
point, the induced and uninduced aliquots (1-ml) of the culture were pelleted down. The  
pellet was boiled in 5X SDS- PAGE loading dye, boiled for 10 minutes and 15 ul of the  
sample was loaded on a 15% SDS- PAGE. Simultaneously as control, the vector alone  
10 (pGEX 5X2) was induced and expressed. Lane 1: Uninduced sample of pGEX 5X2 post  
2 hour of IPTG addition; Lane 2: Induced sample post 2 hour of pGEX 5X2; Lane 3:  
Induced sample of pGEX. MV16 post 2hour IPTG addition; Lane 4: UnInduced samples  
of pGEX MV16 post 2hour IPTG addition; Lane 5: Induced Sample of PGEX-5X2.

Fig 27: SDS- PAGE profile of expression of pGEX- MV16 at 37°C:

15 pGEX MV16 was expressed in BL21DE3λ. The culture was induced with 1mM IPTG  
at 37°C and expression of the protein was studied at various time points. At each time  
point, the induced and uninduced aliquots (1-ml) of the culture were pelleted down. The  
pellet was boiled in 5X SDS- PAGE loading dye, boiled for 10 minutes and 15 ul of the  
sample was loaded on a 15% SDS- PAGE. Simultaneously as control, the vector alone  
20 (pGEX 5X2) was induced and expressed. Lane 1: Induced sample of pGEX\_ MV16  
post 2 hour of IPTG addition; Lane 2: Uninnduced sample of pGEX\_ MV16 post 2 hour  
of IPTG addition; Lane 3: Induced sample of pGEX 5X2 post 2hour IPTG addition;  
Lane 4: Uninduced sample of pGEX 5X2 post 2hour IPTG addition.

Fig. 28: Immunoblot of pGEX- MV16: pGEX- MV16 was expressed in

25 BL21DE3λ. The culture was induced with 1mM IPTG at 30°C. After 2hour of IPTG  
induction, cells were pelleted down. The pellet was boiled in 5X SDS- PAGE loading  
dye, boiled for 10 minutes and 15 µl of the sample was loaded on a 15% SDS- PAGE.  
The proteins were electrophoretically transferred to a nitrocellulose membrane. The  
membrane was blocked with 3% BSA and probed with anti- VIP antibody. Lane 1:  
30 Uninduced pellet of pGEX MV16; Lane 2: Induced pellet of pGEX MV16; Lane 3:  
Uninduced pellet of pGEX Lane 4: Induced pellet of pGEX.

Fig. 29: SDS- PAGE profile of purified fractions of pGEXMV16: pGEX



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MV16 was expressed in BL21DE3 $\lambda$ . The fusion protein was purified on Glutathione-sepharose column. Lane1: Molecular weight marker; Lane 2- 8: Purified fractions

Fig. 30: Immunoblot of purified fractions of pGEXMV16: pGEX MV16 was expressed in BL21DE3 $\lambda$ . The fusion protein was purified on Glutathione-sepharose column. The purified fractions were loaded on a 15% SDS- PAGE. The proteins were electro-phoretically transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA and probed with anti- SP antibody. Lane 1: Uninduced pellet of pGEX MV16; Lane 2: Induced pellet of pGEX MV16; Lane 4-8: Purified fractions.

Fig. 31: SDS- PAGE profile of expression of pET-MVSC1 at 30°C: pET-MVSC1 was expressed in BL21DE3 $\lambda$ . The culture was induced with 1mM IPTG at 30°C and expression of the protein was studied at various time points. At each time point, the induced and uninduced aliquots (1-ml) of the culture were pelleted down. The pellet was boiled in 5X SDS- PAGE loading dye, boiled for 10 minutes and 15 ul of the sample was loaded on a 15% SDS- PAGE. Lane 1: Induced sample post 2 hour of IPTG addition; Lane 2: Uninduced sample post 2 hour; Lane 3: Induced sample post 2 $\frac{1}{2}$  hour of IPTG addition; Lane 4: Uninduced sample post 2 $\frac{1}{2}$  hour.

Fig 32: SDS- PAGE profile of expression of pET-MVSC14 at 30°C: pET- MVSC1 was expressed in BL21DE3 $\lambda$ . The culture was induced with 1mM IPTG at 30°C and expression of the protein was studied at various time points. At each time point, the induced and uninduced aliquots (1-ml) of the culture were pelleted down. The pellet was boiled in 5X SDS- PAGE loading dye, boiled for 10 minutes and 15 ul of the sample was loaded on a 15% SDS- PAGE. Lane 1: Induced sample post 2 hour of IPTG addition; Lane 2: Uninduced sample post 2 hour; Lane 3: Induced sample post 2 $\frac{1}{2}$  hour of IPTG addition; Lane 4: Uninduced sample post 2 $\frac{1}{2}$  hour.

Fig 33: Polyclonal antibody titers to recombinantly expressed multivalent protein and individual peptides: 96- well ELISA plates were coated with 500ng/ well of the multivalent protein and 500ng/well of Bombesin, EGF, SubstanceP and VIP respectively. The non- specific sites were blocked with 3% BSA and subsequently probed with 1:100 dilution of pre- immune serum, boost1 and boost 2 sera respectively. Color was developed with o-phenylenediamine (OPD), and optical density read at 492 nm.



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Fig 34: Immunoblot of tumor lysates with polyclonal antibodies: Tumor cell lysates of breast cancer (MCF-7) and prostate cancer (DU145) were probed with either rat polyclonal antibodies (preimmune or boost2 sera) to the recombinantly expressed multivalent protein (Lane1, 2 and 3), or with anti- SP antibodies (Lane4 and 5) respectively

### DETAILED DESCRIPTION OF INVENTION

The present invention relates to a synthetic multivalent epitope vaccine. For the synthesis of this multivalent vaccine, we identified four peptides viz. VIP, Bombesin, Substance P and EGF, which are overexpressed in cancer. The immunogenic epitopes of each of the four peptides were identified and their aminoacid sequence determined. A multivalent polypeptide sequence was designed with a two aminoacid viz. Glycine-Glycine as linker between each of the four peptides. Following the design of the polypeptide sequence, they were backtranslated into the nucleotide sequence. For this, the prokaryotic codon optimization was used to enable optimal cloning and expression of the multivalent peptide in *E.coli*. Based on the designed multivalent gene sequence, oligonucleotides were synthesized for Splice-Overlap-Extension (SOE) PCR. 70-mer oligonucleotides were synthesized with an overlap region of 20-mer. As described below in the Examples of this invention, the gene coding for synthetic multivalent vaccine was constructed by overlapping PCR technology using oligonucleotides corresponding to the sequence of the designed gene. After each step of PCR, the gene product was purified by gel-elution kit and gene sequencing done to confirm the correct sequence. The complete amplified constructed gene was then cloned into various prokaryotic expression vectors and expressed in the *E.coli* expression system. The protein expression was visualized by SDS-PAGE and the multivalent protein migrated at a molecular weight of 11kDa. The multivalent protein was probed with commercially available antibodies to each of the individual peptides by Western blot, to confirm the presence of the respective peptides in the multivalent sequence. The recombinantly expressed protein was then purified using affinity column chromatography / electroelution / standard chromatographic procedures. The expressed multivalent protein was further used to immunize rats for the production of polyclonal antibodies. The multivalent protein was also studied for its possible role in elucidating a CMI / T-cell response in the murine model.



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The bacterial plasmid vectors which was used for the cloning of the multivalent synthetic vaccine includes the *E. coli* plasmids viz. pGEX, pET, etc. and their derivatives. The pET vectors are derived from pBR322 and vary in leader sequences, expression signals, relevant restriction sites, etc. These vectors are classified  
5 as the transcriptional and translational vectors. The translational vectors, such as pET-20, pET-22, pET-25, etc. contain efficient translational signals and are designed for protein expression. Target genes are cloned under control of strong bacteriophage T7 transcription and translational signals, and providing a source of T7 RNA polymerase in the host cell induces expression. The vector has an ampicillin resistance marker. The  
10 gene is expressed in frame with His residues, which enables one- step purification of the protein by affinity column using Ni- NTA matrix.

The pGEX vectors are widely used for the cloning and expression of genes. The genes are cloned under the control of tac promoter, which is chemically inducible and allows high level expression of the cloned genes. The gene has been  
15 expressed in fusion with the GST protein and thus, can be purified on a glutathione sepharose.

This is one example of a vector that can be used for expression of the multivalent gene and derivatives thereof. Different vectors with various promoters and polyA signals, recombinant viruses, expression vectors for other species such as for  
20 example yeast, fungi, insect cells and plants could be used. A selection of large number of prokaryotic hosts, such as *E.coli* (HM101, BL21, etc.) was used for the expression of the synthetic multivalent gene.

The multivalent protein may be administered serially or in combination with other therapeutics used in the treatment of cancer and other related diseases. These  
25 therapeutics include IFN- alpha, IFN-beta, interleukin-1, interleukin-2, tumor necrosis factor, macrophage colony stimulating factor, macrophage activation factor, lympho-toxin, fibroblast growth factor, etc (derived from natural sources or expressed recombinantly). Alternatively, the synthetic multivalent vaccine may be administered serially or in combination with conventional chemotherapeutic agents such as 5- fluoro  
30 uracil; paclitaxel; etoposide; carboplatin; cisplatin; topotecan, methatroxate, etc. and/ or radiotherapy. Such combination therapies may advantageously utilize less than

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conventional dosages of those agents, or involve less radical regimens, thus avoiding any potential toxicity or risks associated with those therapies.

As per this invention, the recombinantly expressed multivalent vaccine can be administered in any pharmaceutically acceptable form, intratumorally, peritumorally, interlesionally, intravenously, intramuscularly, subcutaneously or by topical routes to exert local therapeutic effects. As an alternative to administering the synthetic multivalent vaccine, the gene encoding the vaccine may be introduced into the cancer cells by treating the infected cells, for example, by scraping them to allow uptake of DNA, by electroporation, by direct injection, etc.

For a better understanding of the invention, the following examples are set forth. Throughout these examples, all molecular cloning reactions was carried out according to methods in Molecular Cloning- A laboratory Manual, 3<sup>rd</sup> Ed., Cold Spring Harbor Press 2001.

#### Reagents & Materials

The following chemicals were used for the experiments- Agarose; Ampicillin; Bovine Serum Albumin; Brilliant blue R250; Bromo phenol blue; Diaminobenzidiamine; Sodiumdodecylsulphate; Glucose; Glycine (Sigma); Ethylenediaminetetraaceticacid; Imidazole; Isopropylthiogalactoside (Calbiochem); Phenylmethylsulfonylfluoride; Sodium dihydrogen phosphate; Disodium hydrogen phosphate; Tris (hydroxymethyl aminomethane); Triton- X-100; Tween20; Urea (Merck); Yeast Extract. Tryptone (Difco); Protease inhibitor tablets (Roche). Goat anti-rat- IgG (HRP-labeled) (SIGMA); Deoxycholate; Q-Sepharose (Amersham Pharmacia).

The reagents used for the experiments were procured from-Anti- EGF antibodies (Sigma, USA; Roche, Germany; Bachem, Switzerland; R&D, USA); Anti-VIP antibodies(Sigma, USA; Bachem, Switzerland); Anti- SP antibodies (Sigma, USA; Bachem, Switzerland; Roche, Germany); Anti- Bombesin (Bachem, Switzerland); Anti-His antibodies (Sigma, USA); Anti- GST antibodies (Sigma, USA); Anti- mouse antibodies (Sigma, USA); Anti- rabbit antibodies (Sigma, USA); Dialysis tubing (Pierce, USA); Glutathione Sepharose (Amersham Pharmacia, UK); Ni- NTA Agarose (Qiagen, USA); Nitrocellulose membrane (Promega, USA); DH5 $\alpha$  cells (Bangalore Genei); BL21DE3 $\lambda$  cells (Novagen, USA); Restriction digestion enzymes and Modifying enzymes(GIBCO BRL, USA; NEB,UK Promega, USA).



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The equipments used for the experiments were- PCR Machine (Perkin Elmer); Incubator shaker (Innova); Tabletop centrifuge (Heraeus); Protein PAGE Apparatus (Bio-Rad); Horizontal gel apparatus (Bangalore Genei); ELISA Reader (Anthos); Spectrophotometer (Shimadzu,).

5

### Example1

#### Construction Of The Synthetic Gene

To construct the synthetic gene, 70-mer oligonucleotide have been designed with an overlap of 20- 25 mer. In the first step of the PCR, MVSV1 (SEQ ID NO:7) and MVSV2 (SEQ ID NO:8) were used. The two primers have an overlap of 20 oligonucleotides. The reaction condition of the PCR was - 97°C for 3min, followed by thirty cycles of 95°C at 1min, 45°C for 2min, 72°C for 30 sec. After this a final step of elongation at 72°C for 7 min was followed by cooling at 4°C. A 126 bp product (as shown in Fig. 3, lane 2) was obtained after the first step. This amplified product was gel eluted (using Qiagen Kit) and used as the template for the second- step PCR.

15 In the second step, MVSV7 (SEQ ID NO:13) and MVSV3 (SEQ ID NO:9) were used as the forward and reverse primers respectively. Product of the first step PCR was used as the template. The PCR conditions were similar to the first step, except for the annealing temperature (55°C for 2 min). A 179-bp product was obtained after second- step PCR, (as shown in Fig, 3, lane 3), which was then similarly gel-  
20 purified and used as the template for the next step.

In the third step, MVSV7 (SEQ ID NO:13) and MVSV4 (SEQ ID NO:10) were used as the forward and reverse primers respectively. Product of the second step PCR was used as the template. The PCR conditions were similar to the first step, except for the annealing temperature (59°C for 2 min). A 232-bp product was  
25 obtained following third- step PCR (as shown in Fig. 3, lane 4), which was then gel purified and used as the template for the following step.

In the fourth step, MVSV7 (SEQ ID NO:13) and MVSV5 (SEQ ID NO:11) were used as the forward and reverse primers respectively. Product of the third step PCR was used as the template. The PCR conditions were similar to the third step.  
30 285 bp product was obtained (as shown in Fig. 3, lane 5), which was gel purified and used as the template for the next step.

In the fifth step, MVSV7 (SEQ ID NO:13) and MVSV6 (SEQ ID

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NO:12) were used as the forward and reverse primers respectively. Product of the fourth step PCR was used as the template. The PCR conditions were similar to the first step, except for the annealing temperature (65°C for 2 min). 336 bp product was obtained (as shown in Fig. 3, lane 6), which was purified and used as the template for the next step.

5 The sixth PCR step was performed with the primers MVSV7 (SEQ ID NO:13) and MVSV8 (SEQ ID NO:14). The primers were of shorter length. This step was performed to obtain a final PCR product, which can then be subsequently used for cloning procedures. The 336bp PCR product was used as the template. The PCR conditions were similar to the fifth step, except for the annealing temperature (62°C for 10 2 min). 336 bp product was obtained (as shown in Fig. 3, lane 7), which was purified and used for next step.

For further cloning of the constructed gene, restriction enzyme sites were incorporated at the 5' and 3' ends in the next step of PCR. MVSV9 (SEQ ID NO:15, having EcoR1 site) and MVSV10 (SEQ ID NO:16, having Not 1 site) were used. PCR 15 conditions were similar as reported in earlier paragraph. 336-bp product was obtained, which was purified and used for next step. Downstream primer with a stop codon (MVSV 11; SEQ ID NO:17) was designed, which enabled the cloning of the multivalent gene in pET vector without the fusion tag

## Example 2

### 20 Description of the vectors

The prokaryotic expression vectors selected for the cloning of the multivalent gene were pET-22b(+), pGEX5X2 and pRSET- A. pET 22b(+) is a 5.5kb prokaryotic vector. The vector has an Ampicillin resistance marker and a multiple cloning site, which allows the selection of appropriate restriction enzyme sites for 25 cloning (Fig. 4). The target genes are cloned under control of strong bacteriophage T7 transcription and translation signals. The protein was expressed in frame with 6 His residues, which enable a one- step purification of the target protein (on a Nickel- NTA column). The His tag was expressed at the C- terminal of the gene.

pGEX 5x-2 is a 4.9kb vector (Fig. 5). The expression of the cloned gene 30 is under the control of *tac* promoter, which is IPTG inducible and allows high level expression of the cloned genes. The protein was expressed as C- terminal fusion with



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GST moiety, which enables the purification of the protein on a glutathione- sepharose column.

pRSET A is a 2.9kb vector (Fig. 6). The target genes are cloned under control of strong bacteriophage T7 transcription and translation signals. The protein was expressed in frame with 6 His residues, which enable a one- step purification of the target protein (On a Nickel- NTA column). The His tag is expressed at the N- terminal of the gene.

### Example 3

#### Cloning Of The Multivalent Synthetic Gene

##### Example3a

#### Cloning Of The Multivalent Synthetic Gene In Pet- 22b(+) Vector:

The plasmid pET 22b (+) was digested with the restriction enzymes EcoR1 and Not1, followed by dephosphorylation using *Calf-alkaline phosphatase* and gel purified to be used as the vector. In a similar manner, the 336bp synthetic gene product (with the EcoR1/ Not1) sites was restricted digested, and gel purified. The vector (pET-22b (+) and the insert (multivalent gene) were ligated in different ratios and transformed in *DH5a* cells. Plasmid isolation of the transformed colonies was carried out (by alkaline lysis method). The positive clones were identified by Restriction enzyme digestion (using EcoR1 and Not1). As seen in Fig. 7, a large number of plasmid clones showed a fall- out of 336bp after the restriction enzyme digestion. Nucleotide sequencing was done for these clones using standard sequencing protocols.

Amongst the clones sequenced, clone #2 (Fig. 8) had 99% identity with the original multivalent sequence. Clone #2 has 2 substitutions towards the C- terminal of the fourth peptide viz. the EGF peptide. (Fig. 9) These substitutions do not effect the immunogenicity or the biological activity of the multivalent peptide as the overall reading frame and the epitopes of the four peptides along with their Gly- Gly linkers have been maintained.

The gene was further expressed and purified on a Nickel- NTA column.

##### Example 3b

#### Cloning of the multivalent synthetic gene in pGEX- 5x2 vector

Following a similar strategy, the multivalent gene was cloned at the

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EcoR1/ Not1 site of the pGEX- 5x2 vector. The clones were screened by Restriction enzyme digestion. A large number of positive clones were obtained (Fig. 10)

Amongst the clones sequenced, clone #16 (Fig. 11) had 98% identity with the original multivalent sequence. The data shows an addition of a nucleotide towards the 3' end of the gene. Consequently, there has been a shift in the reading frame, leading to the inclusion of a stop codon at the 99<sup>th</sup> codon position (Fig 12). As a result the last 14 aa are not expressed. Also, due to nucleotide substitutions, 3 amino acids prior to the stop codon have been changed. These substitutions do not effect the immunogenicity or the biological activity of the multivalent peptide as the overall reading frame and the epitopes of the four peptides along with their Gly- Gly linkers have been maintained.

The protein was further expressed and purified on a glutathione-sepharose column.

### Example 3c

#### Cloning Of The Multivalent Synthetic Gene (With A Stop Codon) In Pet- 22b(+) Vector

As discussed above, the multivalent gene was successfully cloned in pET-22b (+). However, the vector does not have a protease cleavage site by which the tag can be cleaved off from the full-length mature protein. To overcome this, an alternative strategy was followed. Fresh primers were designed with additional stop codon site in the reverse primer. This enables us to amplify our final multivalent synthetic gene of 336 bp with the stop codon incorporated in it. Using this product a similar strategy of cloning in pET-22b (+) was followed. This would enable us to purify the expressed multivalent protein using standard chromatography procedures. Following cloning and screening, a large number of transformed clones were positive (Fig. 13). Clone #1 (Fig. 14, Fig. 15), 9 (Fig. 16, Fig. 17), and 14 (Fig. 18, Fig. 19) were selected after standard DNA sequencing.

### Example 4

#### Expression And Purification Of The Multivalent Protein

Based on the sequencing data, the following clones were selected for



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further studies- clone #2 (pET-mv2, with His tag); clone #16 (pGEX- mv16, with GST tag); clone # 1, 9 and 14 (pET-SC 1, 9 and 4, respectively with a stop codon immediately after the gene sequence).

#### Example 4a

##### 5 Expression Of Clone #2 (Pet- Mv2)

##### Induction And Expression Of The Protein

pET- mv2 was transformed in the expression system *E.coli* BL21DE3 $\lambda$ .

Standard procedure of the induction of target protein expression was followed.

Induction of protein expression was standardized for two different temperatures, viz.

10 30°C and 37°C. For optimizing the temperature and time period of induction, mini-scale protein expression was studied. A single colony was inoculated in 5ml LB broth (containing 100  $\mu$ g/ ml Ampicillin) and incubated overnight at 37°C / in an incubator shaker with aeration at 220 rpm. Inoculated 5 ml of LB medium containing 100  $\mu$ g/ml Ampicillin with 100  $\mu$ l of the overnight culture. Following inoculation, cultures were  
15 incubated at 37°C / 220rpm until the optical density of the culture at 600nm was 0.6.

Transferred 1 ml of the uninduced culture (zero- time aliquot) to microfuge tubes.

Induced the remainder of the culture by adding IPTG to a final concentration of 1mM and continued incubation at 30°C for 3 hours. At various time points during the

induction period (eg. ½, 1, 1½, 2, 2½ and 3hour), transferred aliquot of the cultures  
20 (both the induced and the uninduced cultures). After each time point, cells of each culture was pelleted down. Each pellet was resuspended in 100  $\mu$ l of 1X SDS gel-loading buffer, boiled the samples for 5 min and loaded on a 15% analytical SDS-PAGE gel (Fig. 20). Following a similar methodology, the time- kinetics of induction

was carried out at 37° C for a period of 3 hours and subsequently analyzed on a 15%  
25 analytical SDS- PAGE gel (Fig.21). Comparing Fig. 27 and 28, it was concluded that the induction of the multivalent protein was optimal at 30°C with an induction period of 2½ hours.

Large-scale induction and expression of the pET- MV2 clone was done.

Immunoblot of the crude extract with antibodies against the peptides comprising the

30 multivalent protein viz., anti- VIP, anti- EGF, and anti- Substance-P further confirmed the expression of the multivalent protein. Following SDS- PAGE, the proteins were electrophoretically transferred on the nitrocellulose membrane and non-specific sites

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blocked with 3% BSA. The membrane was then probed with each of the anti- peptide antibodies, followed by peroxidase conjugated secondary antibodies. Protein band of the expected size was seen in the induced crude extract samples (Fig. 22). As control, uninduced, sample was probed with anti-SP antibody (Fig 22, lane 5). Immunoblot of the crude extract with anti- His antibodies (Fig. 22, lane 2) ensured the expression of the full- length protein.

#### **Purification Of The Multivalent Protein (With His- Tag)**

The expression of the multivalent protein in fusion with a C- terminal poly-histidine tag enables one step purification of the protein on a  $\text{Ni}^{3+}$ - NTA column.

The poly- histidine tag is poorly immunogenic and at a pH of 8.0, the tag is small, uncharged, and therefore generally does not affect the secretion, compartmentalization, or folding of the fusion protein within the cell. The protein purification on the  $\text{Ni}^{2+}$ - NTA column (Qiagen) was done under denaturing as well as native conditions.

#### **Purification Under Native Conditions**

The cell pellet was resuspended (from 100ml culture) in 4ml of binding buffer [50 mM  $\text{NaH}_2\text{PO}_4$ ; 300m NaCl; 10mM imidazole; pH 8.0], containing 1X protease inhibitor cocktail and 1% Triton- X-100. The cell solution was sonicated (in ice) for 3 min, followed by centrifugation at 13,000 rpm for 15 min. at 4° C. The Ni- NTA resin was equilibrated with the lysis buffer and this  $\text{Ni}^{2+}$ - NTA slurry was applied to the cell lysate, mixed gently by shaking at 4°C for 30 min, and then loaded the lysate- Ni-NTA mixture on a column and the flow- through collected. The column was washed thoroughly with the wash buffer [50mM  $\text{NaH}_2\text{PO}_4$ , 300m NaCl; 10mM imidazole; pH 8.0], and the protein eluted with the elution buffer [50mM  $\text{NaH}_2\text{PO}_4$ , 300m NaCl; 300mM imidazole; pH 8.0]. The histidine residues in the 6X H is tag has imidazole ring in the side chain that interacts with the Ni ions on the column. If the imidazole concentration in the elution buffer is increased to 100- 300mM, the 6X His-tagged protein would diassociate because they can no longer compete for binding sites on the Ni- NTA resin. Fractions of 500  $\mu\text{l}$  were collected, and loaded on 15% analytical SDS- PAGE. Following SDS- PAGE, the proteins were electrophoretically transferred on the nitrocellulose membrane whose non-specific sites were blocked with 3% BSA. The membrane was then probed with anti- SP antibody. (Fig 23).



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Purification Using Standard Chromatography

The cell pellet was resuspended (from 100ml culture) in 50mM Tris buffer, pH 8.5. The cell suspension was sonicated (in ice) for 8 cycles 1 minutes/cycle, followed by centrifugation at 12,000 rpm for 10 min. at 4°C. The pellet was  
5 resuspended in 50mM Tris buffer, pH 8.5 containing 2% Deoxycholate. The cell suspension was sonicated (in ice) for 8 cycles, 1 minute/cycle, followed by centrifugation at 12,000 rpm for 10 min. at 4°C. The pellet was resuspended in MilliQ water, and further centrifuged at 12,000 rpm for 10 min. at 4°C. The pellet was solubilized in 100mM Tris buffer, pH 8.5 containing 2M Urea. The solution was made  
10 alkaline by increasing the pH to above 10, centrifuged at 12,000 rpm/10min/ 4°C. The supernatant was diluted to 5 times the original volume and pH decreased to 8.5. The sample was loaded on a Q sepharose column (pre- equilibrated with 20mM Tris, pH 8.5, 2M Urea). The column was washed with 3 volumes of equilibration buffer and eluted with increasing concentration of NaCl (in equilibration buffer- upto 1M NaCl). The  
15 eluted samples were checked on a 15% SDS- PAGE gel.

**Electroelution Of The Multivalent Protein**

Expression of the pET- MV2 clone results in a full- length multivalent protein with a 6- His tag. The 6 His tag being very small in size does not interfere with the structure and function of the protein and can be used for downstream applications.  
20 In a parallel experiment, electroelution of the protein from SDS- PAGE was done. The crude extract of the induced pET-MV2 sample was loaded on a 15% preparative gel. Electrophoresis was done at constant voltage of 100V for about 3hrs. Two longitudinal strips were cut from the right and left side of the gel, stained with Coomassie blue. After destaining, the stained side strips were aligned with the rest of the gel and used as  
25 guides to cut out bands from the unstained gel.

The gel strip was equilibrated in three changes of 2mM-phosphate buffer, pH 6.8. This removes the toxic components- tris, glycine, SDS, etc. The gel strip was cut into small pieces, sealed in dialysis tubing (M.w. cut off- 3,500) with 2mM phosphate buffer, pH 6.8 to just cover the gel slices. Dialysis tubing was transferred to  
30 the platform of horizontal gel electrophoresis apparatus filled with 2mM-phosphate buffer. Electroelution was carried out at 40 V for 40 min at 4°C. Significant amount of protein sticking to dialysis tubing was desorbed by reversal of current for 1 min. towards

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the end of main elution period. The protein sample was taken out from the dialysis bag and was concentrated in a lyophilizer, under cold conditions. It was reconstituted in minimal volume of 2mM PBS and analyzed on a 15% SDS-PAGE gel. The protein moved as a single band of approximately 11kDa on SD- PAGE (Fig. 24). Immunoblot of the protein with the individual anti- peptide antibodies showed sharp band, a representative with the anti- Substance P antibodies as seen in Fig. 25, Lane 1 and 2.

Here, the multivalent protein was successfully expressed as His tag fusion protein, and was purified by affinity column chromatography as well as directly electroeluted from the gel. This protein is being used for further characterisation studies.

### **Example 4b**

#### **Expression Of Clone #16 (PGEX- Mv 16)**

##### **Induction and expression of the protein**

Following a methodology similar to that for pET- mv2, the induction of protein expression was standardized for two different temperatures, viz. 30°C and 37°C. Fig. 26 and Fig. 27 shows the SDS- PAGE profile of the time- kinetics study. From the profile it was concluded that the induction of the multivalent protein was optimal at 30°C with an induction period of 2 hours.

Large-scale induction and expression of the pGEX-mv16 clone was done. Immunoblot of the crude extract with anti- peptide antibodies confirmed the expression of the multivalent protein (Fig. 28). As controls, uninduced sample and induced sample of the vector alone was taken.

##### **Purification of the multivalent protein (with GST- tag)**

The expression of the multivalent protein in fusion with a N- terminal GST tag enables one step purification of the protein on a glutathione- sepharose column. Bound GST fusion proteins are readily displaced from the column by elution with buffers containing free glutathione.

Resuspended the cell pellet (from 100ml culture) in 4ml of phosphate-buffered saline, containing 1X protease inhibitor cocktail and 1% Triton- X-100. To the cell suspension lysozyme was added (final concentration of 1mg/ml) and incubated for 30 min, followed by addition of 10 ml of 0.2% Triton-X-100, with thorough mixing. Next, Dnase and RNase were added to a final concentration of 5 µg/ml, and the



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incubation was continued for 10min at 4°C. To the supernatant added DTT to a final concentration of 1mM. As an alternative to lysozyme treatment, the cell lysis was done by sonicating the cells (in ice) for 3min. The cell lysate (prepared in either way) was mixed with PBS- equilibrated glutathione- sepharose, and the mixture incubated for 30 min. at room temperature. The lysate- glutathione-resin mixture was then loaded on a column, and the flow- through collected, and column washed thoroughly with PBS. The protein was eluted with the glutathione- elution buffer [20mM reduced glutathione in 50mM Tris- HCl (pH 8.0)]. SDS- PAGE analysis of the GST column purified fractions shows faint bands corresponding to the fusion protein (Fig. 29), [cell lysate prepared by sonication of cells] was almost similar. The purified fractions were further characterized by immunoblot using the individual anti- peptide antibodies (Fig. 30).

#### **Example 4c**

##### **Expression Of Clones #1, 9 And 14 (Pet- 22b Mv 16)**

##### **Induction And Expression Of The Protein**

All the three clones, viz. pET-mvsc1, pET-mvsc 9 and pET-mvsc14 were transformed in the expression system *E.coli* BL21DE3λ. Standard procedure of the induction of target protein expression was followed. Following a methodology similar to that for pET- mv2, the induction of protein expression was standardized for two different temperatures, viz. 30° C and 37°C. Fig. 31 and Fig. 32 shows the SDS- PAGE profile of the time- kinetics study for the clones pET-mvsc1, and pET-mvsc14 respectively, no protein expression was not seen in clone #9. From the profile it was concluded that the induction of the multivalent protein was optimal at 30°C with an induction period of 2½ hours for both pET-mvsc1 and pETmvsc14.

Large-scale induction and expression of the pET-mvsc 1 and 14 was done. Standard conventional purification techniques would be followed for the purification of the protein.

#### **Example 5**

##### **Production Of Polyclonal Antibodies In Murine Model**

The recombinantly expressed multivalent protein was used to raise polyclonal antibodies in rats. 4-6 weeks old male Wistar rats, obtained from National Institute of Nutrition, Hyderabad were used. On arrival the rats were kept in quarantine for a week before use. After a week, pre-immune blood was collected and rats were

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immunized by s.c. route at a dose of 15-20ug / rat with the multivalent protein. Three weeks post immunization a booster dose of the multivalent protein was administered in a similar fashion. Rats were immunized with 15ug of multivalent protein s.c on the left flank. Two weeks post-boost, the rats were bled from the retro-orbital vein and serum separated. The animals were given a periodic boost of the multivalent protein and bleeds collected. Production of specific immune response to the multivalent protein was observed using standard ELISA technique. The polyclonal antibodies generated in the rats were titrated using ELISA. 96-well ELISA plates were coated with either multivalent protein or each of the individual peptides. (Fig. 33).

### Example 6

#### Elucidation Of Immune Response

Established cancer cell lines such as DU145 (Prostate cancer) and MCF-7 (Breast cancer) were used to study the immune response to the multivalent polyclonal antibodies, raised in rats. Cells were cultured *in vitro* using appropriate culture medium and standard growing conditions, to confluency. Cells were trypsinised and  $2 - 3 \times 10^6$  cells were taken for the study. Cells were suspended in 0.1 M PBS and sonicated for 5 minutes. Following sonication, it was centrifuged at 13,000rpm for 20 minutes at 4°C. The supernatant was collected and used for all further studies. Protein estimation (Bradford reagent) of the culture supernatant was carried out and appropriate amount of the supernatant was used to load on SDS-PAGE gel, followed by Western Blot. The polyclonal antibodies to the recombinantly expressed multivalent protein recognized tumor cell antigens of DU145 and MCF-7 cancer cells, as visualized by immunoblot studies. Further, the tumor cell lysates were also probed with the commercially available anti-peptide antibodies (Fig 34).

#### VASOACTIVE INTESTINAL PEPTIDE (VIP)

##### SEQ ID NO:1

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH<sub>2</sub>

#### BOMBESIN (BOM)

##### SEQ ID NO:2

Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met



- 27 -

**SUBSTANCE P (SP)****SEQ ID NO:3**

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met

**EPIDERMAL GROWTH FACTOR (EGF)**

5

**SEQ ID NO:4**

Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-  
 Asp-Gly-Val-Cys-Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-  
 Cys-Val-Val-Gly-Tyr-Ile-Gly-Glu-Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-  
 Trp-Trp-Glu-Leu-Arg

10

**Designed Sequence for Synthetic Multivalent Cancer Vaccine****Total number of amino acids = 112****SEQ ID NO:5**

15

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
 Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-Gly-Gly-Glu-Gln-  
 Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-Gly-Arg-Pro-  
 Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-Gly-Gly-Asn-Ser-Asp-Ser-Glu-  
 Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-Asp-Gly-Val-Cys-Met-  
 Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-Gly-Tyr-  
 Ile-Gly-Glu-Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-Trp-Trp-Glu-Leu-Arg

20

**Nucleotide Sequence of Multi-Valent Vaccine****SEQ ID NO:6**

25

His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr
CAT	AGC	GAT	GCG	GTG	TTT	ACC	GAC	AAC	TAT
Thr	Arg	Leu	Arg	Lys	Gln	Met	Ala	Val	Lys
ACG	CGT	CTG	CGC	AAA	CAG	ATG	GCC	GTT	AAG
Lys	Tyr	Leu	Asn	Ser	Ile	Leu	Asn	Gly	Gly
AAA	TAC	TTA	AAT	TCG	ATT	CTG	AAC	GGC	GGT
Glu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val
GAA	CAA	CGT	CTT	GGC	AAT	CAG	TGG	GCG	GTG
Gly	His	Leu	Met	Gly	Gly	Arg	Pro	Lys	Pro
GGG	CAC	TTA	ATG	GGC	GGG	CGG	CCG	AAA	CCA

30

- 28 -

Gln Gln Phe Phe Gly Leu Met Gly Gly Asn  
 CAA CAG TTC TTT GGT CTG ATG GGA GGC AAC  
 Ser Asp Ser Glu Cys Pro Leu Ser His Asp  
 AGT GAT AGC GAG TGT CCG CTT TCG CAT GAC  
 5 Gly Tyr Cys Leu His Asp Gly Val Cys Met  
 GGT TAT TGC TTG CAT GAT GGG GTT TGT ATG  
 Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys  
 TAC ATC GAA GCA CTG GAC AAG TAT GCG TGC  
 Asn Cys Val Val Gly Tyr Ile Gly Glu Arg  
 10 AAC TGT GTG GTC GGC TAC ATT GGT GAA CGT  
 Cys Gln Tyr Arg Asp Leu Lys Trp Trp Glu Leu Arg  
 TGC CAG TAT CGC GAT TTG AAA TGG TGG GAA CTG CGC

**Sequences of Designed Oligo's:**

MVSV 1: (1-73) CDS (SEQ ID NO:7)

15 5' CAT AGC GAT GCG GTG TTT ACC GAC AAC TAT ACG CGT  
 CTG CGC AAA CAG ATG GCC GTT AAG AAA TAC TTA AAT T3'

MVSV 2: (53-126) NCDS (SEQ ID NO:8)

5' CCC CCA CCG CCC ACT GAT TGC CAA GAC GTT GTT  
 CACCGC CGT TCA GAA TCG AAT TTA AGT ATT TCT TAA  
 20 CGG3'

MVSV 3: (106-179)NCDS (SEQ ID NO: 9)

5' TTG CCT CCC A TC AGA CCA AAG AAC TGT TGT GGT TTC  
 GGC CGC CCG CCC A TT AAG TGC CCC ACC GCC CAC TGA T  
 T3'

MVSV 4: (159-232) NCDS (SEQ ID NO:10)

5' ACC CCA TCA TGC AAG CAA TAA CCG TCA TGC GAA AGC  
 GGA CAC TCG CTA TCA CTG TTG CCT CCC ATC AGA CCA  
 AAG3'

MVSV 5: (212-285) NCDS (SEQ ID NO:11)

30 5' GCC GAC CAC ACA GTT GCA CGC ATA CTT GTC CAG TGC  
 TTC GAT GTA CAT ACA AAC CCC ATC ATG CAA GCA ATA  
 AC3'



- 29 -

MVSV 6: (265-336) NCDS (**SEQ ID NO:12**)

5' GCG CAG TTC CCA CCA TTT CAA ATC GCG ATA CTG GCA  
ACG TTC ACC AAT GTA GCC GAC CAC ACA GTT GCA CGC 3'

MVSV 7: (1-20) CDS (**SEQ ID NO:13**)

5 5' CAT AGC GAT GCG GTG TTT AC 3'

MVSV 8: (317-336) NCDS (**SEQ ID NO:14**)

5' GC GCA GTT CCC ACC ATT TCA 3'

MVSV 9: CDS (**SEQ ID NO:15**)

5' GAA TTC GAA TTC ATG CAT AGC GAT 3'

10 MVSV 10 : NCDS (**SEQ ID NO:16**)

5' AAT GCG GCC GCT AGC GCA GTT 3'

MVSV 11 : NCDS (**SEQ ID NO:17**)

5' AAT GCG GCC GCC TGA GCG CAG 3'

- 30 -

CLAIMS

1. A multivalent vaccine comprising four peptides; vasoactive intestinal peptide, whose sequence is His-Ser-Asp-Ala -Val -Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met -Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH<sub>2</sub> SEQ ID NO:1), the peptide Bombesin whose sequence is Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met (SEQ ID NO:2), the peptide Substance P whose sequence is Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met (SEQ ID NO:3), and the peptide Epidermal growth factor whose sequence is Asn Ser Asp Ser Glu Cys Pro Leu Ser His Asp Gly Tyr Cys Leu His Asp Gly Val Cys Met Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys Asn Cys Val Val Gly Tyr Ile Gly Glu Arg Cys Gln Tyr Arg Asp Leu Lys Trp Trp Glu Leu Arg (SEQ ID NO:4).
2. The vaccine according to claim 1, wherein SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4 are linked together.
3. The vaccine according to claim 1, wherein SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4 are linked together through a linker comprising of at least two amino acids.
4. The vaccine according to claim 3, wherein the linker is Gly-Gly.
5. A vaccine comprising the amino acid sequence of the linked polypeptide consisting of VIP, Bombesin, Substance P and EGF whose sequence is SEQ ID NO:5.
6. A nucleotide sequence that is optimized to the *E.coli* codons, whose sequence is SEQ ID NO:6.
7. A method for SOE ing PCR, whose product is 126 base pairs.
8. A method for SOE ing PCR, whose product is 179 base pairs.
9. A method for SOE ing PCR, whose product is 232 base pairs.
10. A method for SOE ing PCR, whose product is 285 base pairs.
11. A method for SOE ing PCR, whose complete gene sequence is made up of 336 base pairs.
12. A method for SOE ing PCR product of 336 base pairs, with restriction sites EcoR1 and Not1.
13. Clone # 16 (pGEX-MV) whose sequence comprises the sequence of claims 1, 2, 3 and 4.

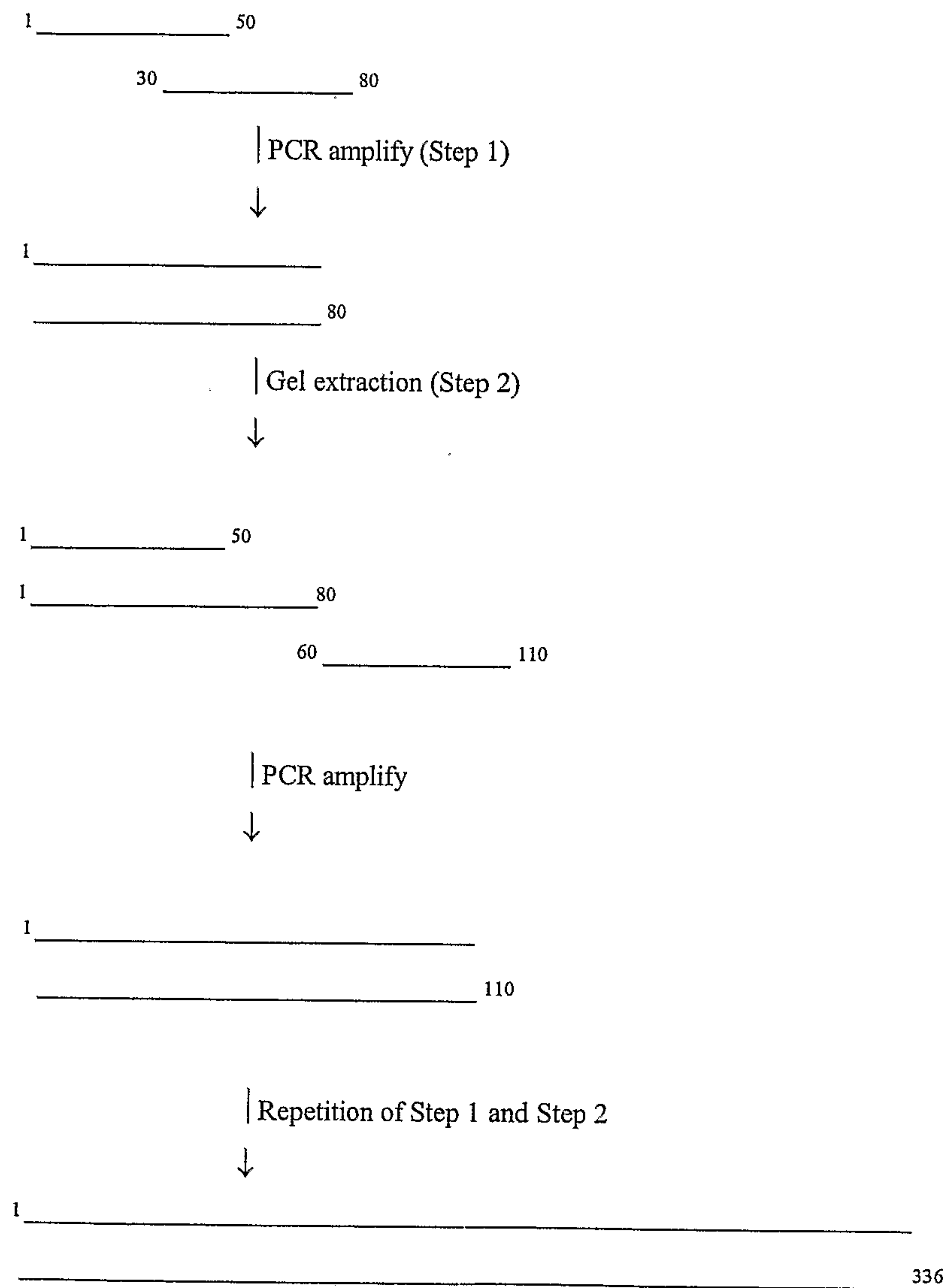


- 31 -

14. Clone # 2 (pET-MV) whose sequence comprises the sequence of claims 1, 2, 3 and 4.
15. Clone # 1, 9 and 14 (pET-MV: SC) whose sequence comprises the sequence of claims 1, 2, 3 and 4.
- 5 16. A recombinantly expressed protein of clone # 16.
17. A recombinantly expressed protein of clone # 2.
18. A recombinantly expressed protein of clone # 1, 9 and 14.
19. The protein as in any one of claims 16 to 18, which is immunogenic and induces a polyclonal antibody response in rats.
- 10 20. A multivalent protein according to any one of claims 16 to 18, which is able to generate a T-cell response in the murine model.

FIG. 1

PCR Strategy



In the final PCR product (obtained after the 10<sup>th</sup> cycle of overlapping PCR), Restriction Enzyme recognition sites will be incorporated at the 5' and the 3' end.



FIG. 2

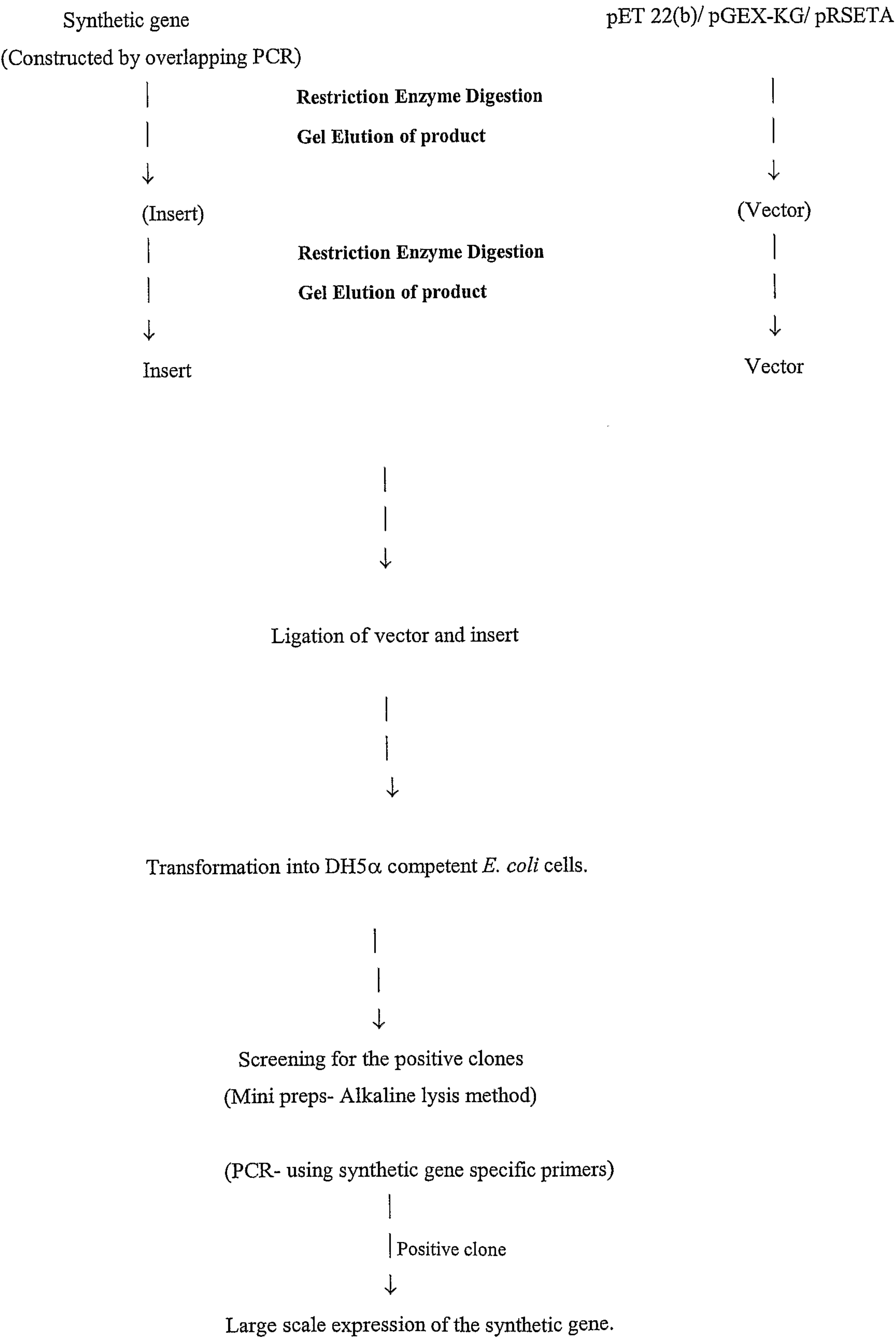
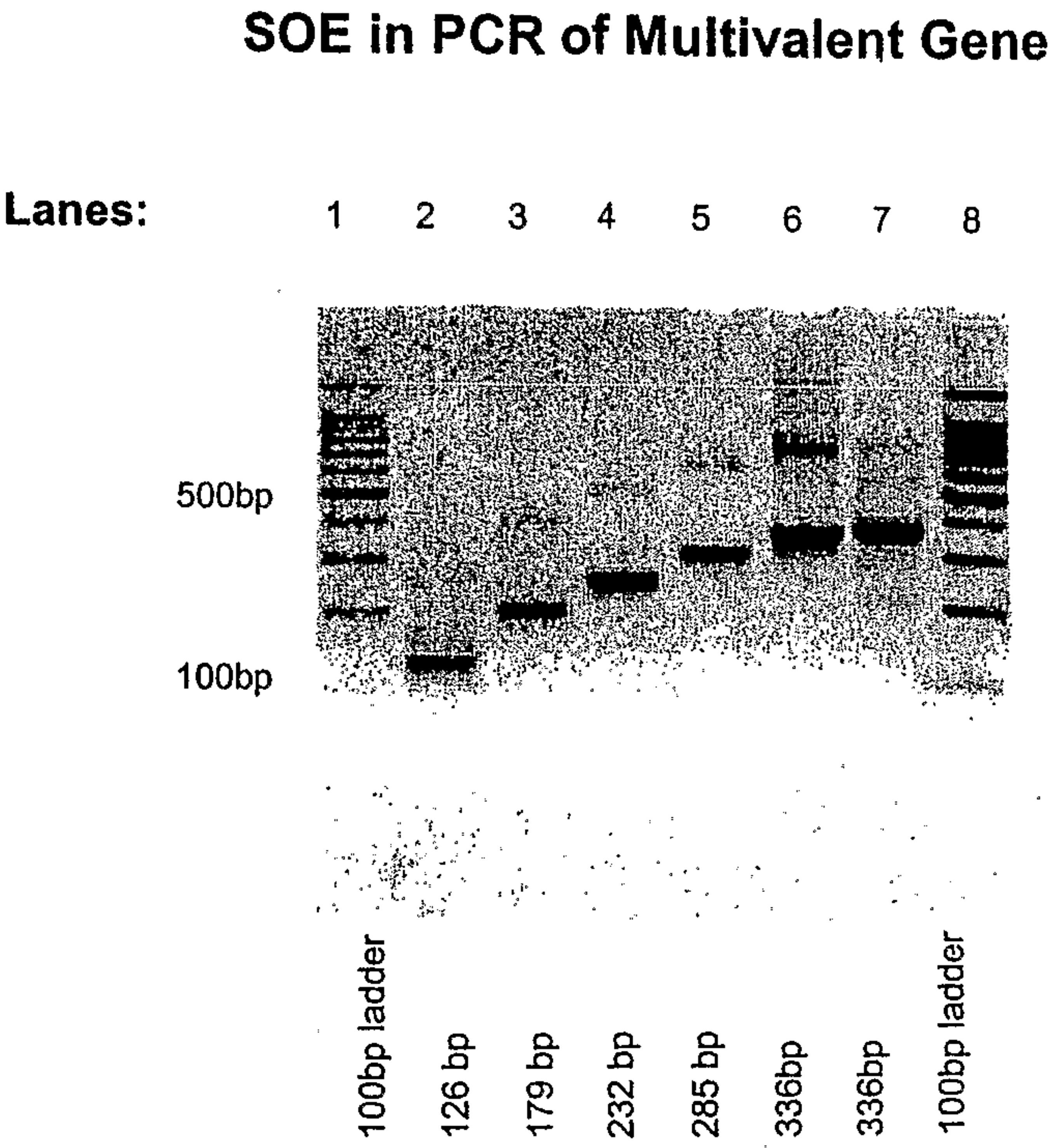


FIG. 3





**pET-22b(+)** Vector

**pET-22b(+)** sequence landmarks

**pET-22b(+) cloning/expression region**

**FIG. 5**

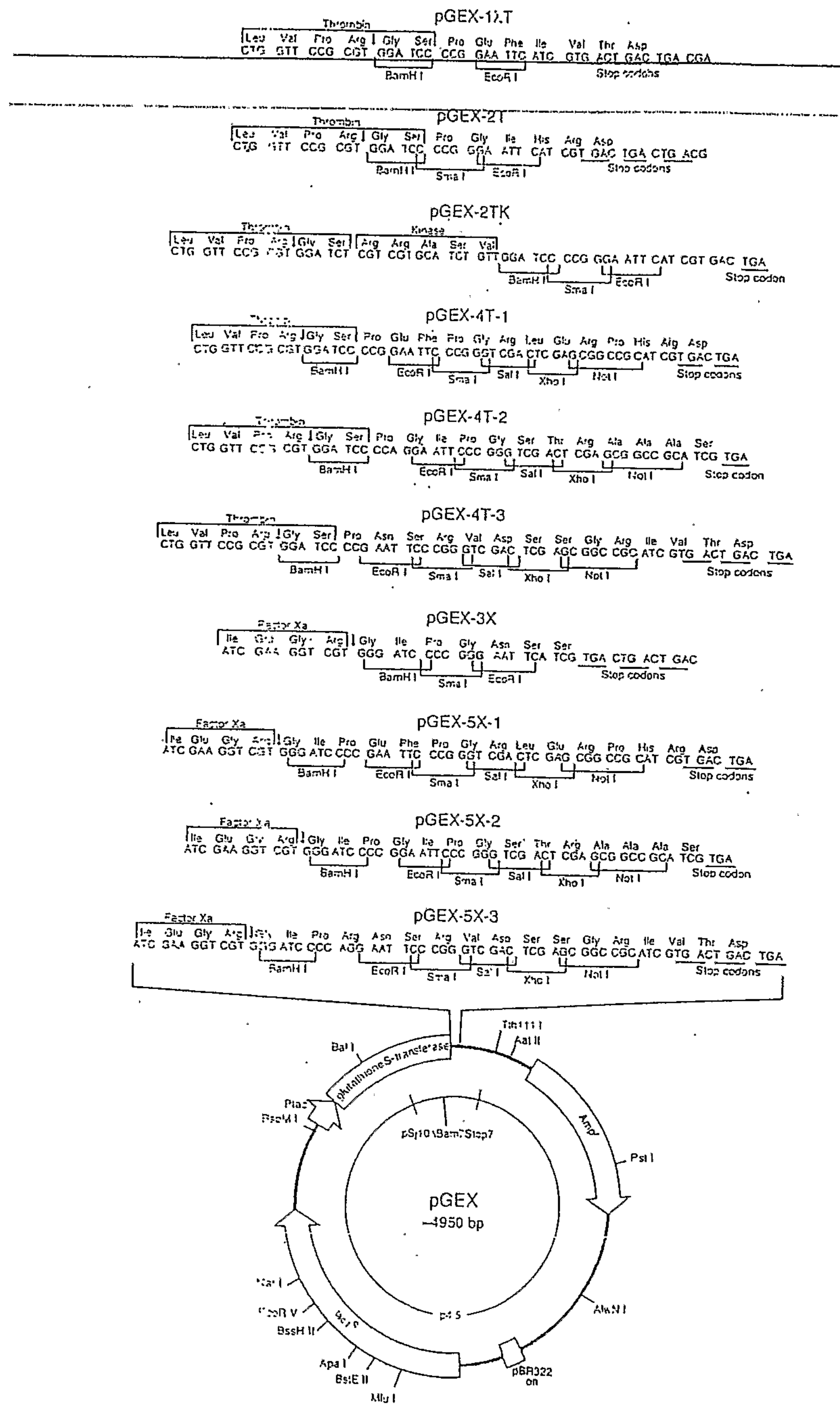
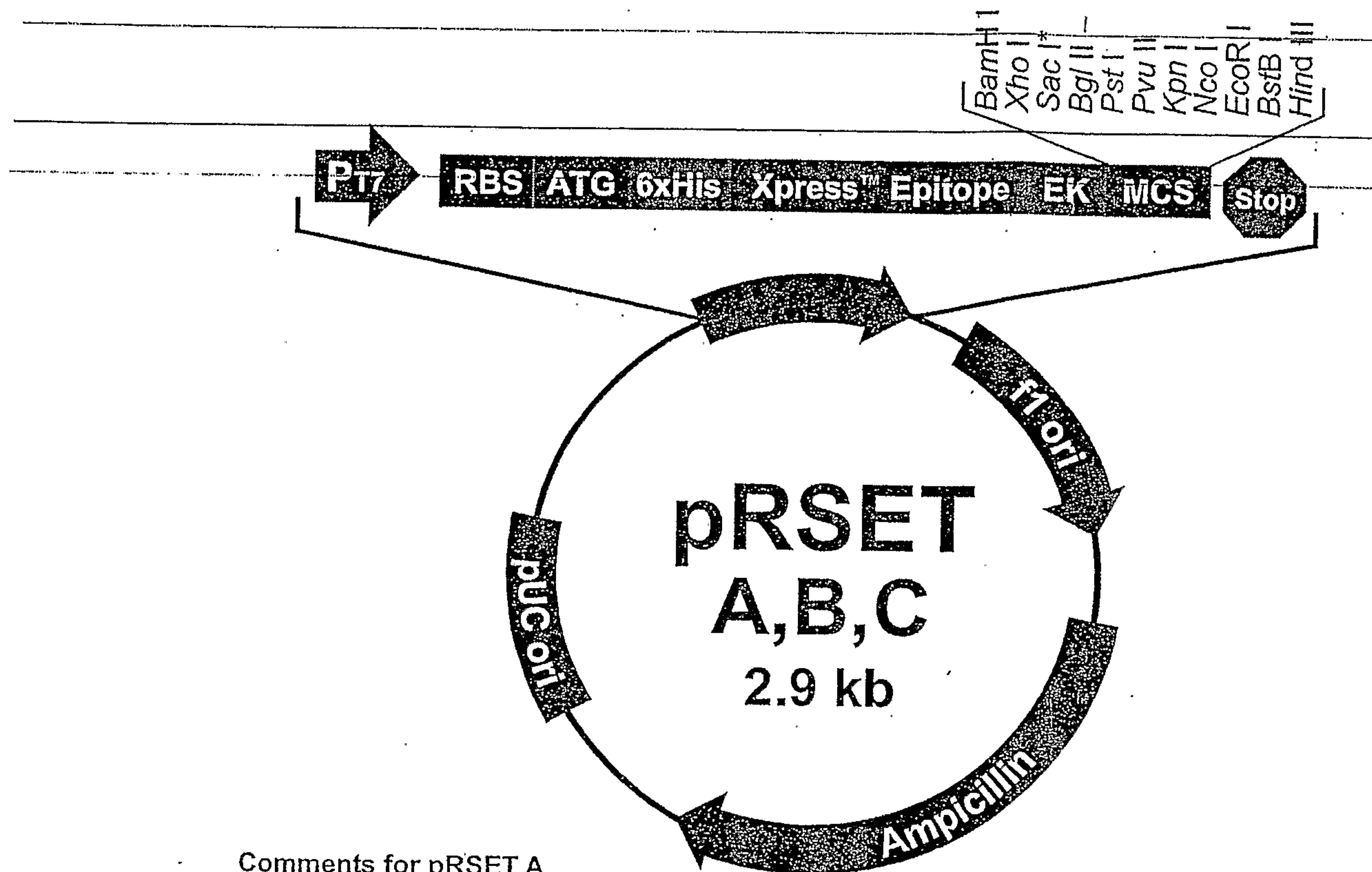




FIG. 6

## Map of pRSET A, B, and C



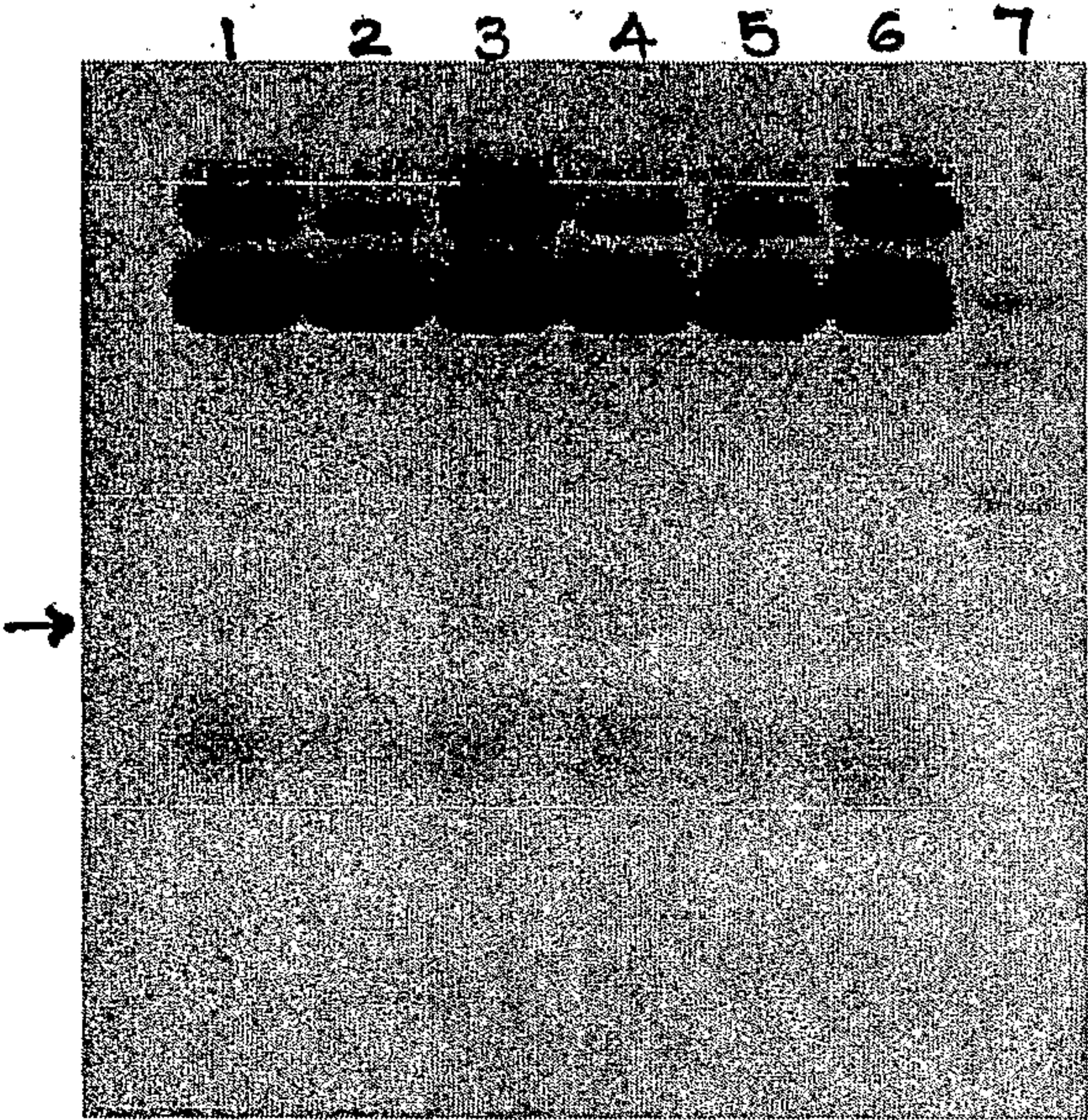
Comments for pRSET A  
2897 nucleotides

\*Version C does not contain Sac I

T7 promoter: bases 20-39  
 6xHis tag: bases 112-129  
 T7 gene 10 leader: bases 133-162  
 Xpress™ epitope: bases 169-192  
 Multiple cloning site: bases 202-248  
 T7 reverse priming site: bases 295-314  
 T7 transcription terminator: bases 256-385  
 f1 origin: bases 456-911  
 bla promoter: bases 943-1047  
 Ampicillin (bla) resistance gene (ORF): bases 1042-1902  
 pUC origin: bases 916-2852 (C)



FIG. 7





**FIG. 8**  
**SEQ ID NO: 18**

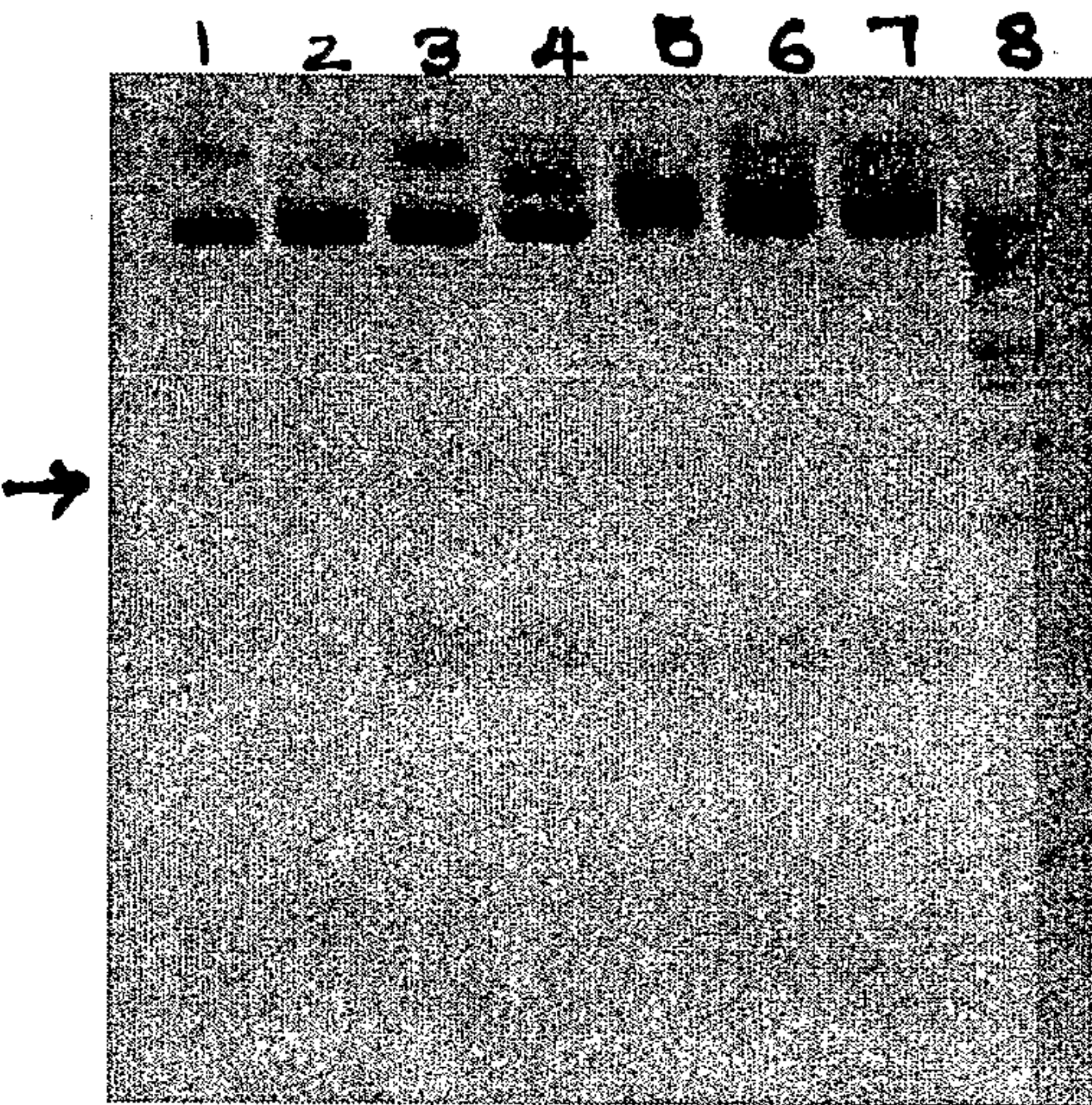
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ctg	cgc	aaa	cag	atg	gcc	gtt	aag	aaa	tac	tta	aat
tcg	att	ctg	aac	ggc	ggt	gaa	caa	cgt	ctt	ggc	aat
cag	tgg	gcg	gtg	ggg	cac	tta	atg	ggc	ggg	cgg	ccg
aaa	cca	caa	cag	ttc	ttt	ggt	ctg	atg	gga	ggc	aac
agt	gat	agc	gag	tgt	ccg	ctt	tcg	cat	gac	ggt	tat
tgc	ttg	cat	<b>ggt</b>	ggg	gtt	tgt	atg	tac	atc	gaa	gca
ctg	gac	aag	tat	gcg	tgc	aac	tgt	gtg	<b><u>a</u>tc</b>	ggc	tac
att	ggt	gaa	cgt	tgc	cag	tat	cgc	gat	ttg	aaa	tgg
tgg	gaa	ctg	cgc								

**FIG. 9****SEQ ID NO: 19**

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-Gly-Gly-Glu-Gln-  
Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-Gly-Arg-Pro-  
Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-Gly-Gly-Asn-Ser-Asp-Ser-  
Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-**Gly**-Gly-Val-Cys-  
Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-**Ser**-  
Tyr-Ile-Gly-Glu-Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-Trp-Trp-Glu-  
Leu-Arg



FIG. 10



**FIG. 11**  
**SEQ ID NO: 20**

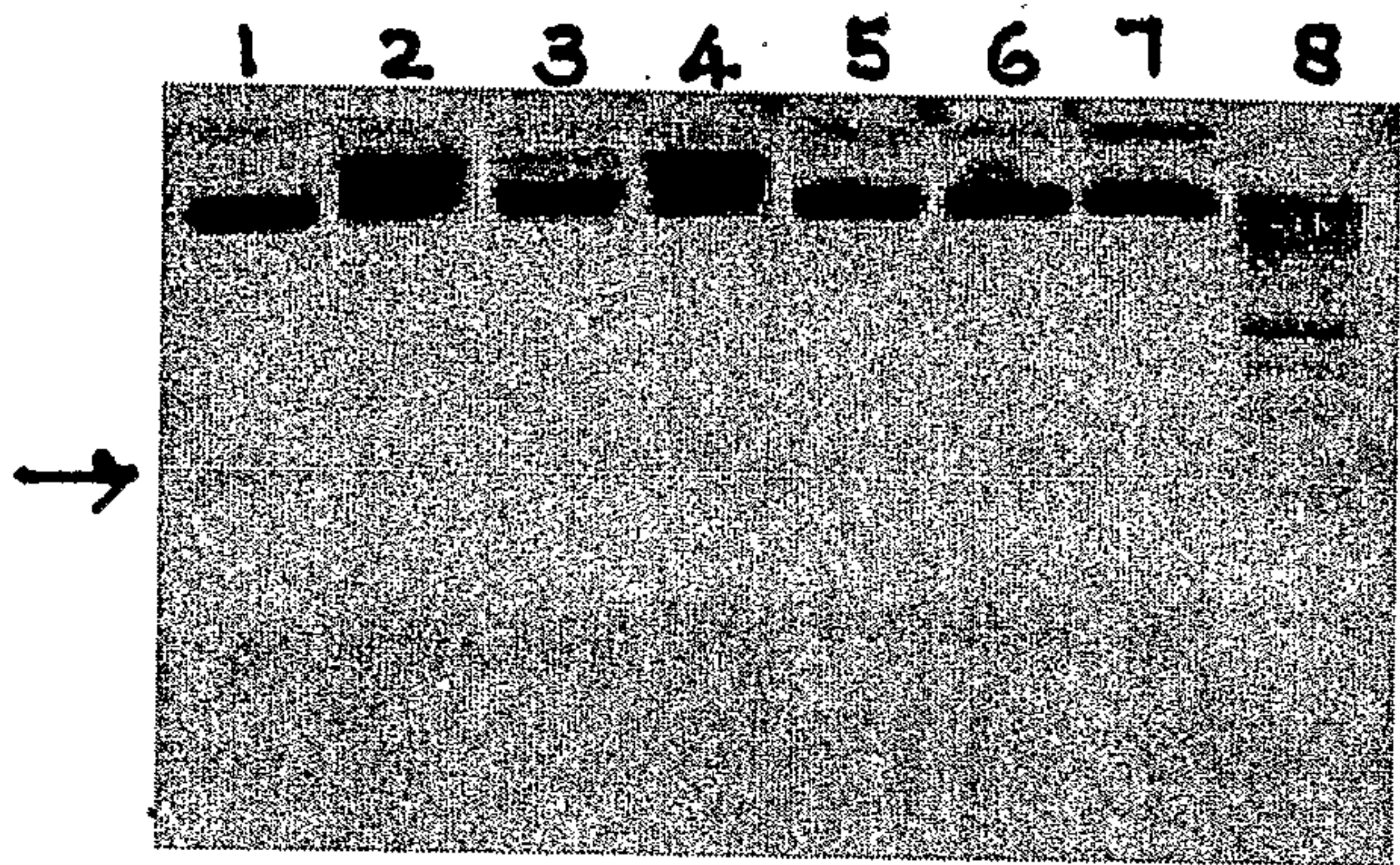
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ctg	cgc	aaa	cag	atg	gcc	gtt	aag	aaa	tac	tta	aat
tcg	att	ctg	aac	ggc	ggg	gaa	caa	cgt	ctt	ggc	aat
cag	tgg	gcg	gtg	ggg	cac	tta	atg	ggc	ggg	cgg	ccg
aaa	cca	caa	cag	ttc	ttt	ggg	ctg	atg	gga	ggc	aac
agt	gat	agc	gag	tgt	ccg	ctt	tcg	cat	gac	ggg	tat
tgc	ttg	cat	gat	ggg	gtt	tgt	atg	tac	atc	gaa	gca
ctg	gac	aag	tat	gcg	tgc	aac	tgt	gtg	gtc	<u>ggg</u>	<u>ata</u>
cat	<u>gtg</u>	tga	acg	ttg	cca	gta	tgn	cgg	t		



**FIG. 12**  
**SEQ ID NO: 21**

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-Gly-Gly-Glu-Gln-  
Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-Gly-Arg-Pro-  
Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-Gly-Gly-Asn-Ser-Asp-Ser-  
Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-Asp-Gly-Val-Cys-  
Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-Gly-  
**Ile-His- Val -stop- Thr-Leu-Pro- Val -X- Arg**

FIG. 13





**FIG. 14**  
**SEQ ID NO: 22**

cat	agc	gat	gcg	gtg	ttt	acc	gac	aac	tat	acg	cgt
ctg	cgc	aag	cag	atg	gcc	gtt	aag	aaa	tac	tta	aat
tcg	att	ctg	aac	ggc	ggt	gaa	caa	cgt	ctt	ggc	aat
cag	tgg	gcg	gtg	ggg	cac	tta	atg	ggc	ggg	cgg	ccg
aaa	cca	caa	cag	ttc	ttt	ggt	ctg	atg	gga	ggc	aac
agt	gat	agc	gag	tgt	ccg	ctt	tcg	cat	gac	ggt	tat
tgc	ttg	cat	gat	ggg	gtt	tgt	atg	tac	atc	gaa	gca
ctg	gac	aag	tat	gcg	tgc	aac	tgt	gtg	gtc	ggc	tac
att	ggt	gaa	cgt	tgc	cag	tat	cgc	gat	ttg	aaa	tgg
tgg	gaa	ctg	cgc								

**FIG. 15**  
**SEQ ID NO: 23**

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-Gly-Gly-Glu-Gln-  
Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-Gly-Arg-Pro-  
Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-Gly-Gly-Asn-Ser-Asp-Ser-  
Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-Asp-Gly-Val-Cys-  
Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-Gly-  
Tyr-Ile-Gly-Glu-Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-Trp-Trp-Glu-  
Leu-Arg



**FIG. 16**  
**SEQ ID NO: 24**

cat	agc	gat	gcg	gtg	ttt	acc	gac	aac	tat	acg	cgt
ctg	cgc	aaa	cag	atg	gcc	gtt	aag	aaa	tac	tta	aat
tcg	att	ctg	aac	ggc	ggg	gaa	caa	cgt	ctt	ggc	aat
cag	tgg	gcg	gtg	ggg	cac	tta	atg	ggc	ggg	cgg	ccg
aaa	cca	caa	cag	ttc	ttt	ggg	ctg	atg	gga	ggc	aac
agt	gat	agc	gag	tgt	ccg	ctt	tcg	cat	gac	ggg	tat
tgc	ttg	cat	gat	ggg	gtt	tgt	atg	tac	atc	gaa	gca
ctg	gac	aag	tat	gcg	tgc	aac	tgt	gtg	gtc	ggc	tac
att	ggg	gaa	cgt	tgc	cag	tat	cgc	gat	ttg	aaa	tgg
tgg	gaa	ctg	cgc								

**FIG. 17**  
**SEQ ID NO: 25**

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-Gly-Gly-Glu-Gln-  
Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-Gly-Arg-Pro-  
Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-Gly-Gly-Asn-Ser-Asp-Ser-  
Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-Asp-Gly-Val-Cys-  
Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-Gly-  
Tyr-Ile-Gly-Glu-Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-Trp-Trp-Glu-  
Leu-Arg



**FIG. 18**  
**SEQ ID NO: 26**

cat	agc	gat	gcg	gtg	ttt	acc	gac	aac	tat	acg	cgt
ctg	cgc	aaa	cag	atg	gcc	gtt	aag	aaa	tac	tta	aat
tcg	att	ctg	aac	ggc	ggg	gaa	caa	cgt	ctt	ggc	aat
cag	tgg	gcg	gtg	ggg	cac	tta	atg	ggc	ggg	cgg	ccg
aaa	cca	caa	cag	ttc	ttt	ggg	ctg	atg	gga	ggc	aac
agt	gat	agc	gag	tgt	ccg	ctt	tcg	cat	gac	ggg	tat
tgc	ttg	cat	gat	ggg	gtt	tgt	atg	tac	atc	gaa	gca
ctg	gat	aag	tat	gcg	tgc	aac	tgt	gtg	gtc	ggc	tac
att	ggg	gaa	cgt	tgc	cag	tat	cgc	gat	ttg	aaa	tgg
tgg	gaa	ctg	cgc								

**FIG. 19**  
**SEQ ID NO: 27**

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-Gly-Gly-Glu-Gln-  
Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-Gly-Arg-Pro-  
Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-Gly-Gly-Asn-Ser-Asp-Ser-  
Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-Asp-Gly-Val-Cys-  
Met-Tyr-Ile-Glu-Ala-Leu-**Glu**-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-Gly-  
Tyr-Ile-Gly-Glu-Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-Trp-Trp-Glu-  
Leu-Arg



FIG. 20



FIG. 21

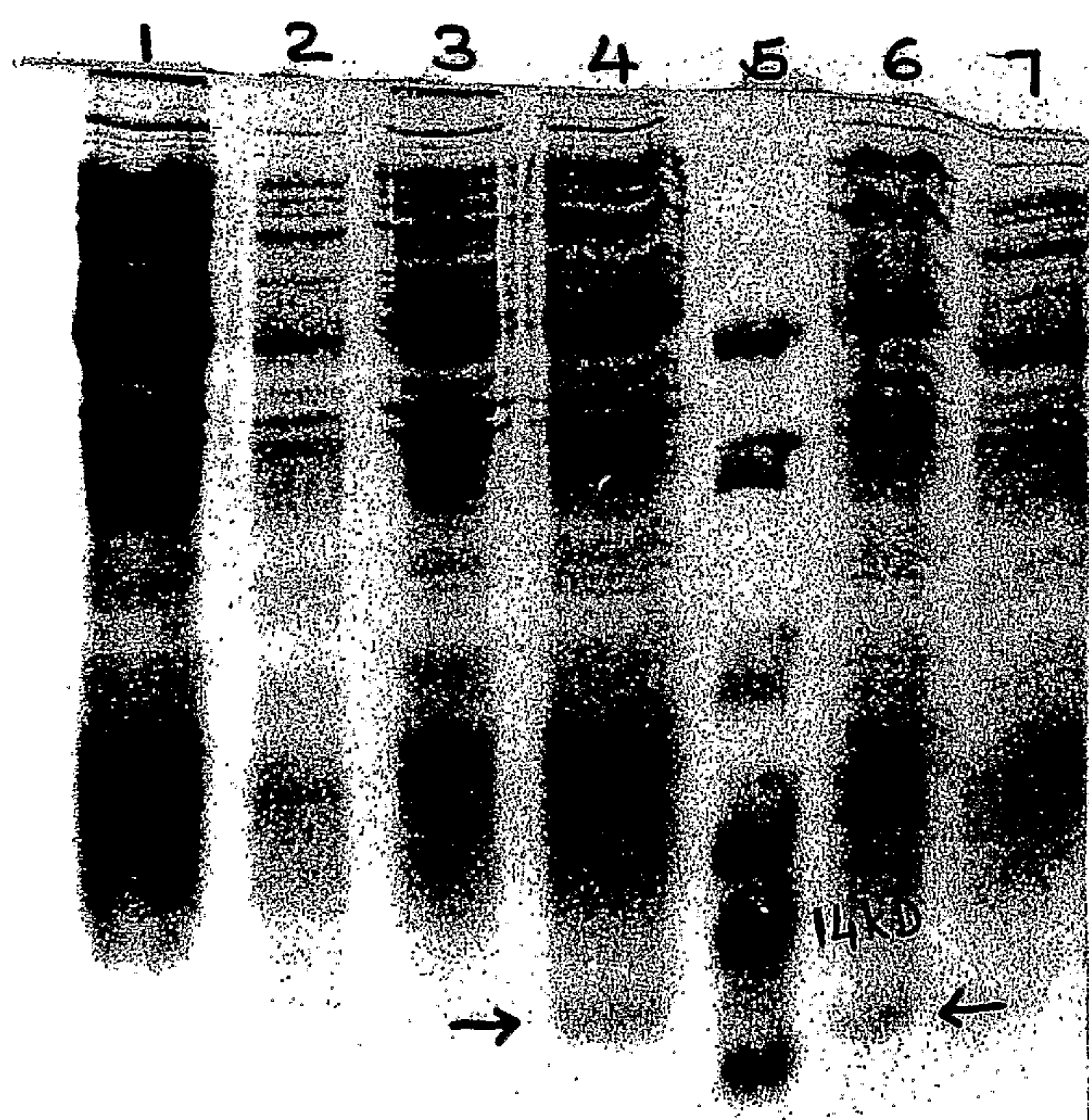




FIG. 22

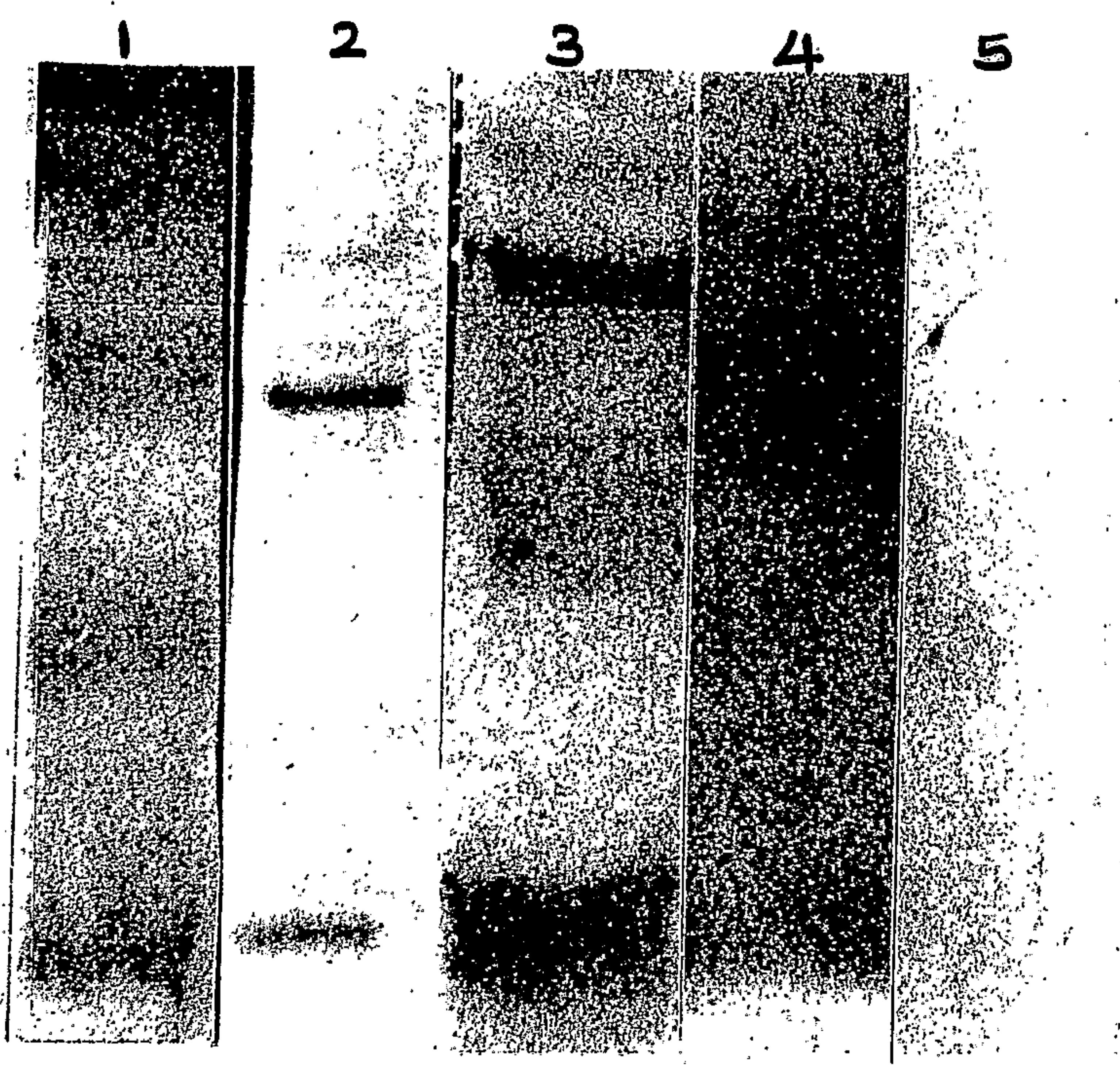


FIG. 23





FIG. 24

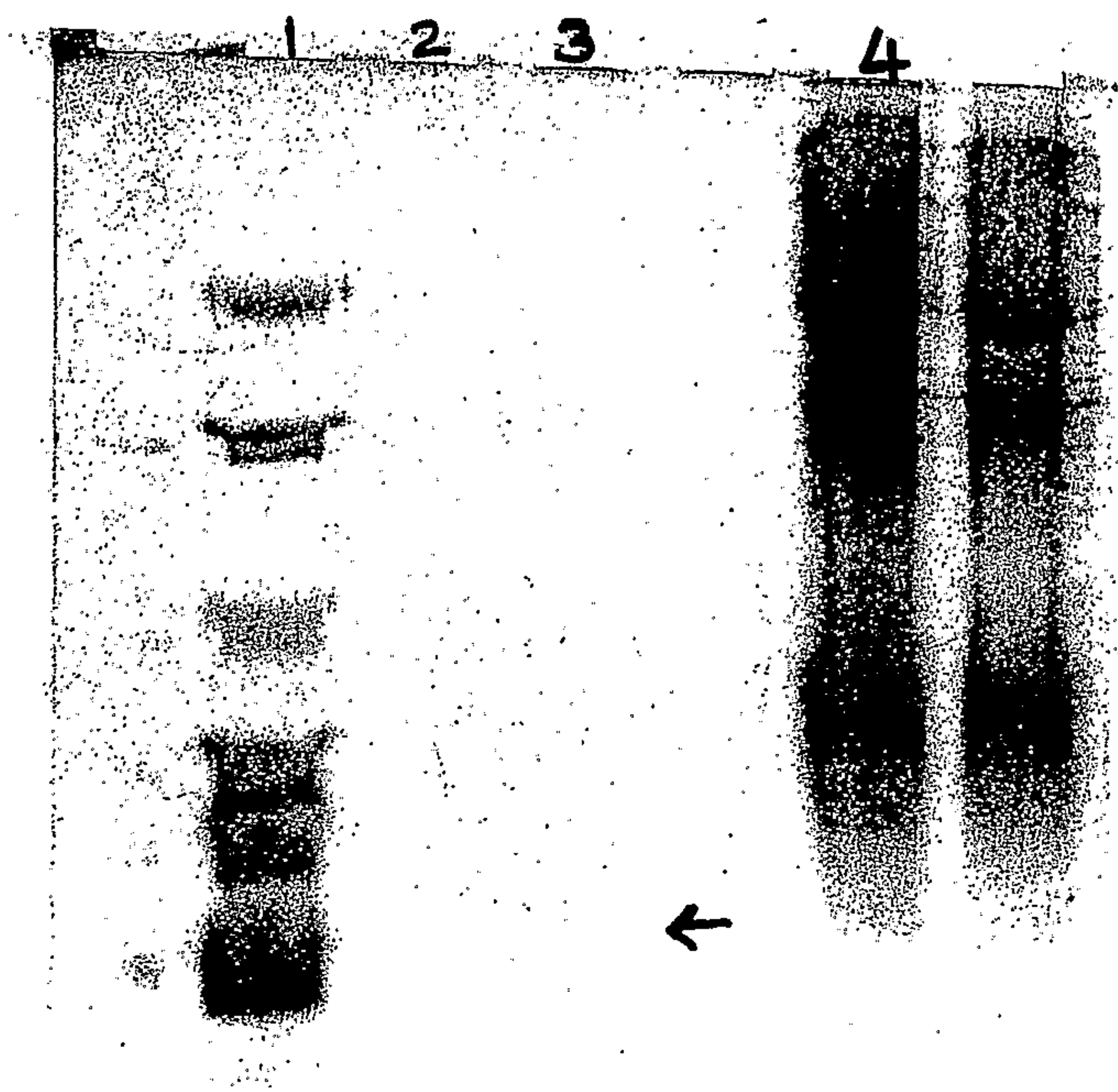


FIG. 25A

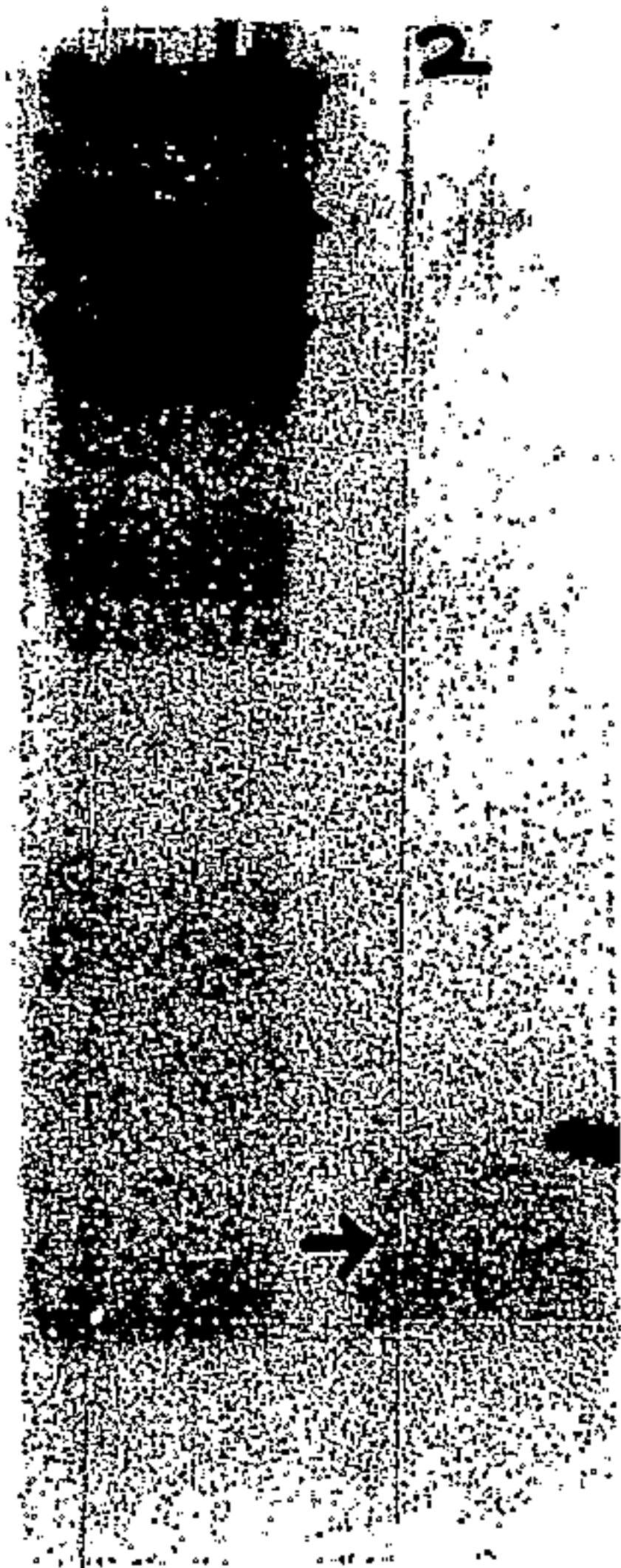


FIG. 25B





FIG. 26

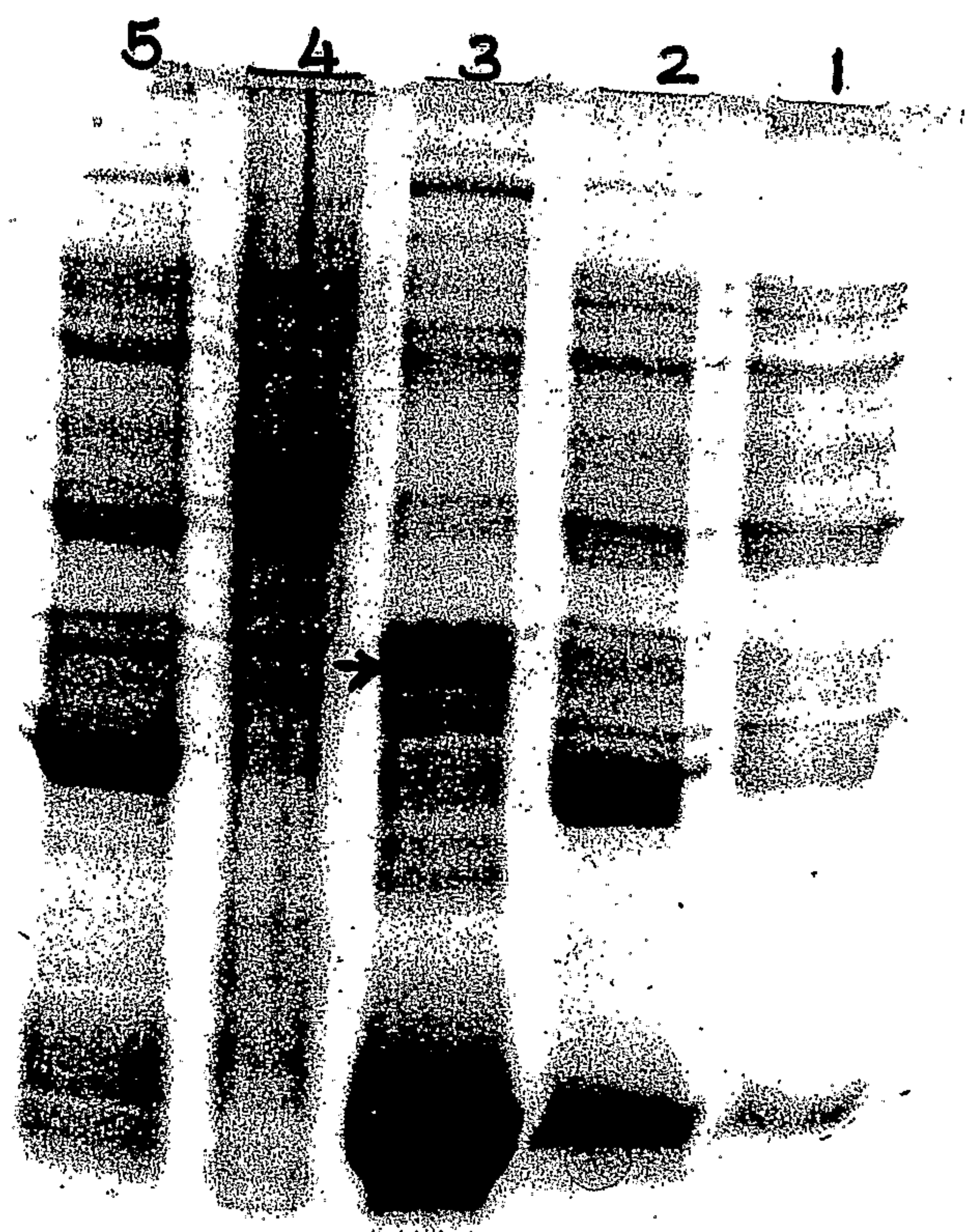


FIG. 27

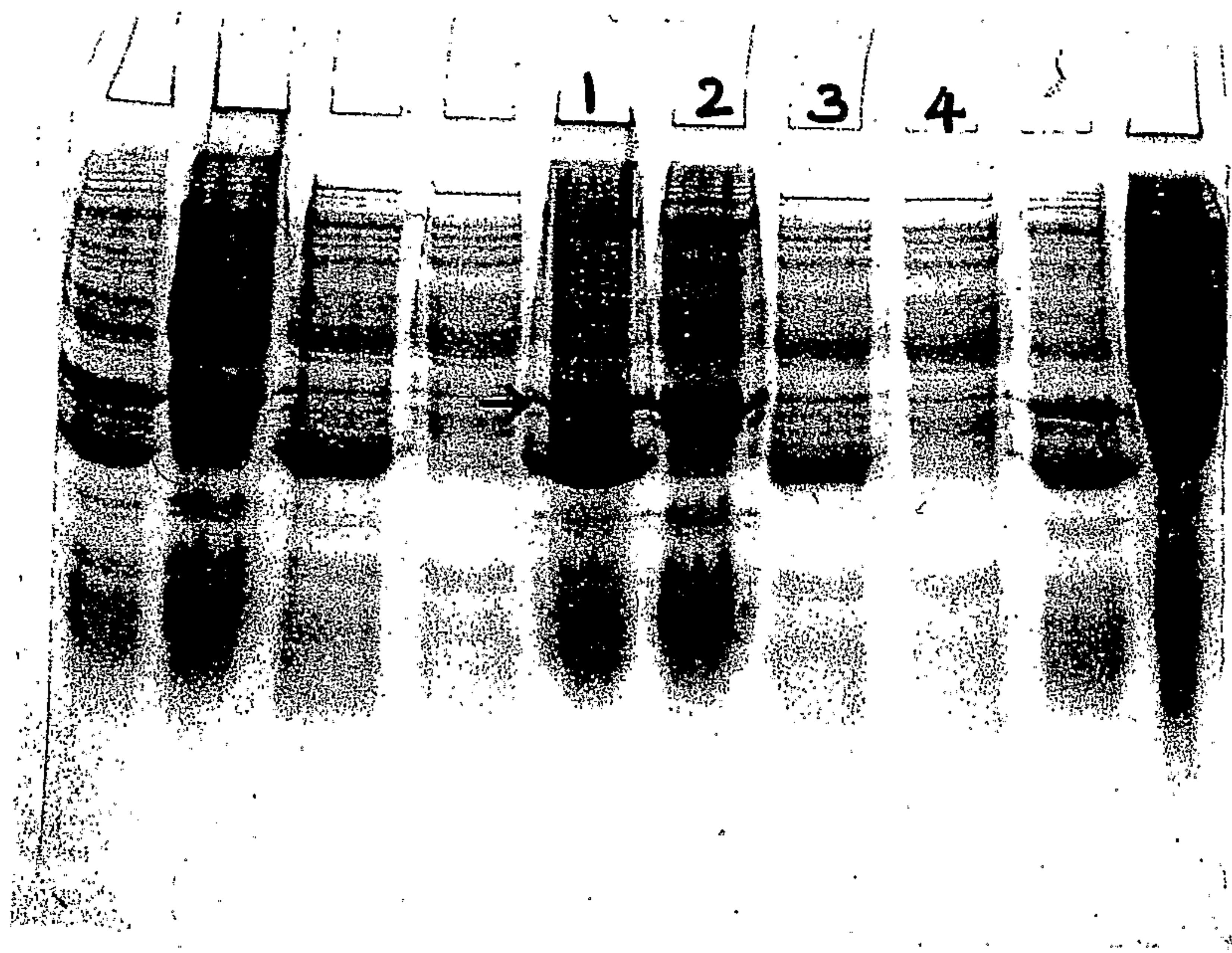




FIG. 28

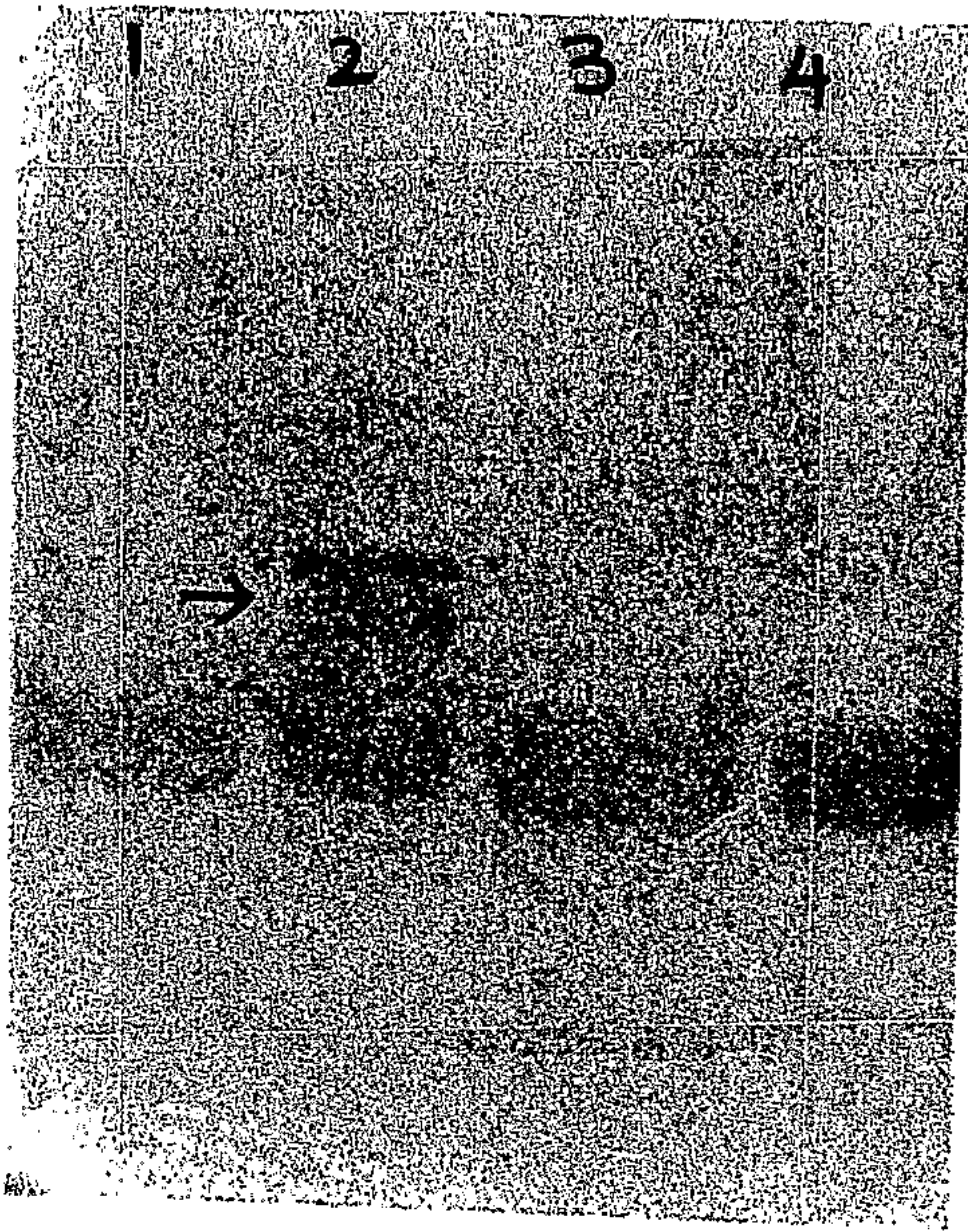


FIG. 29

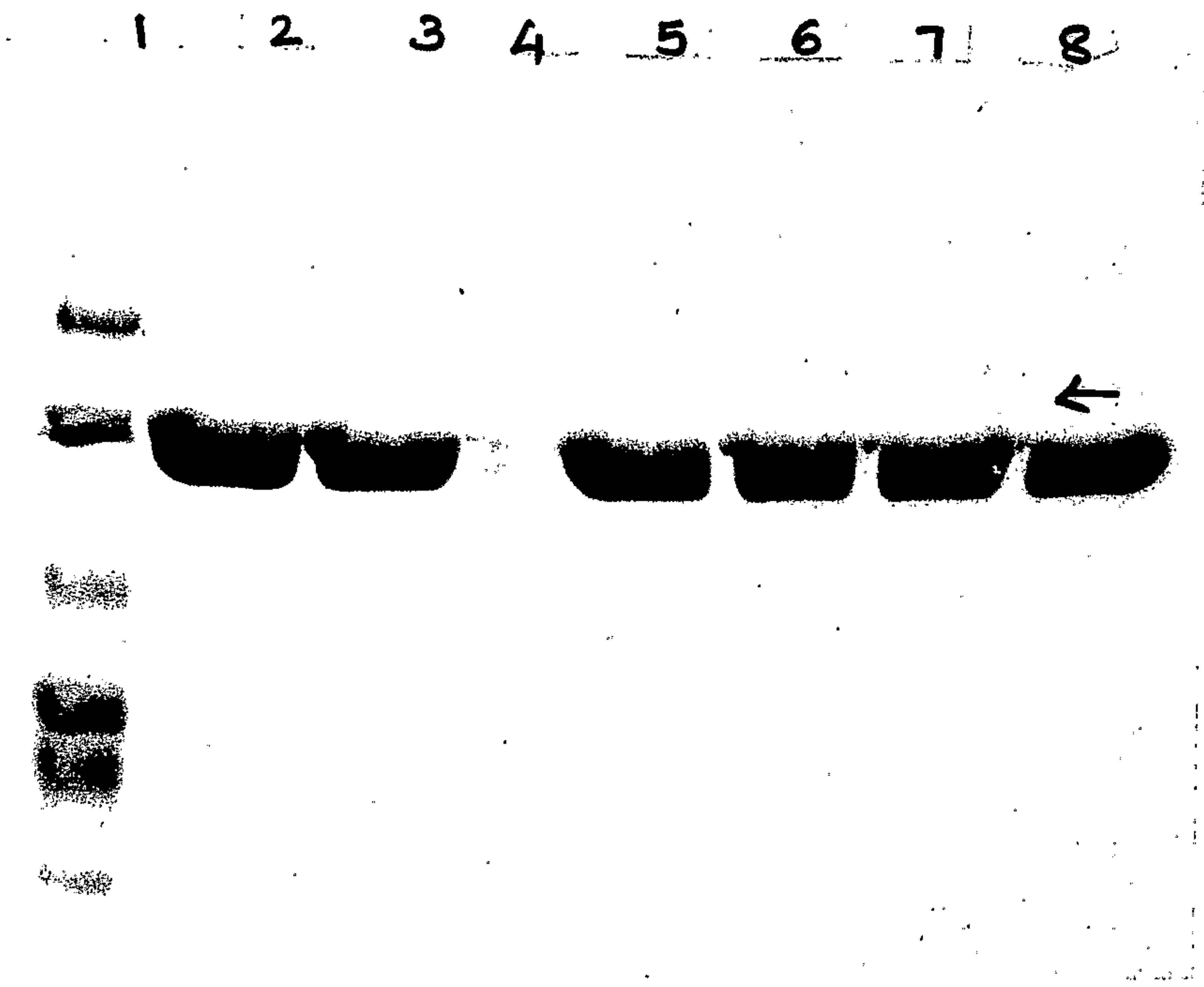




FIG. 30

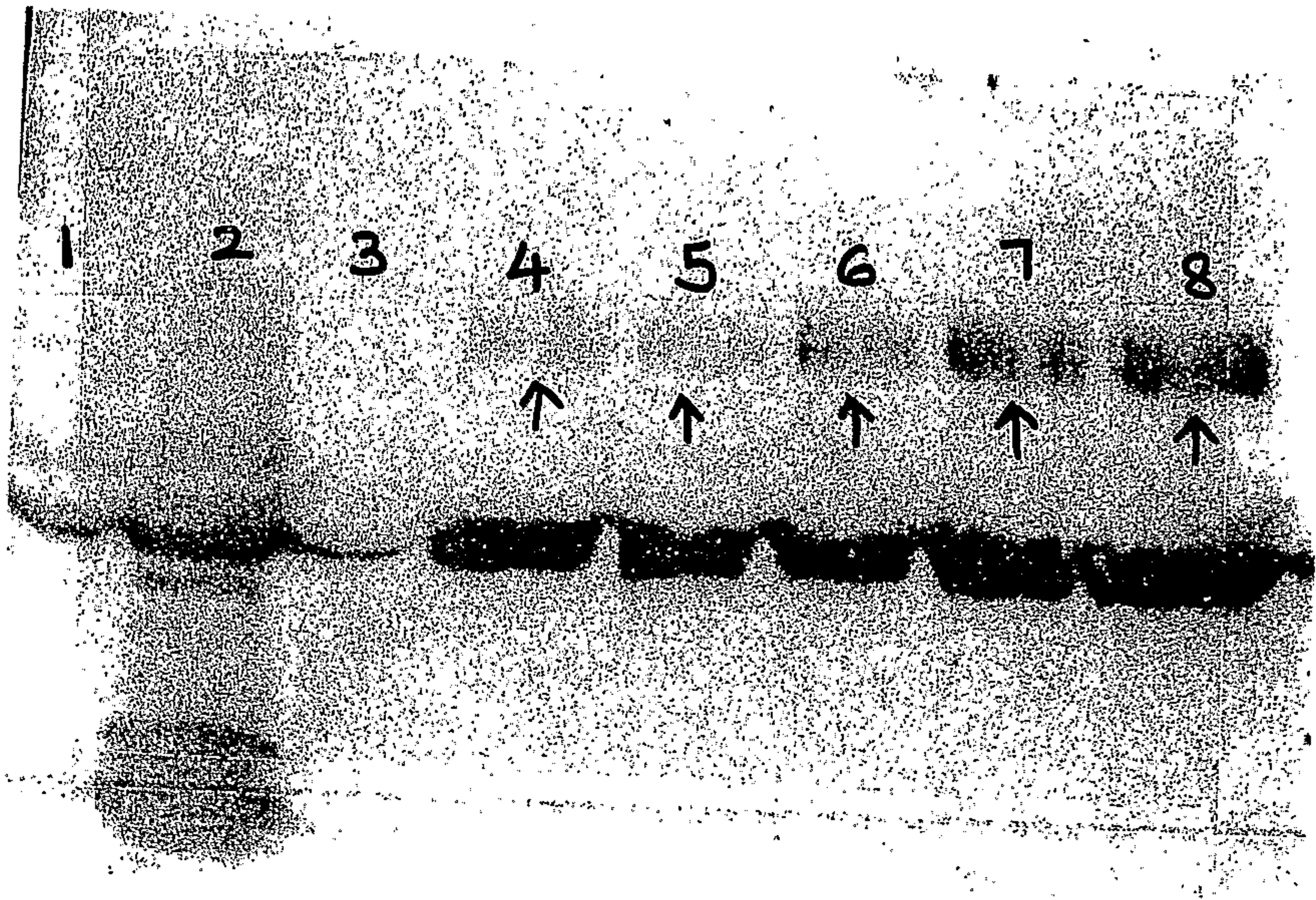


FIG. 31

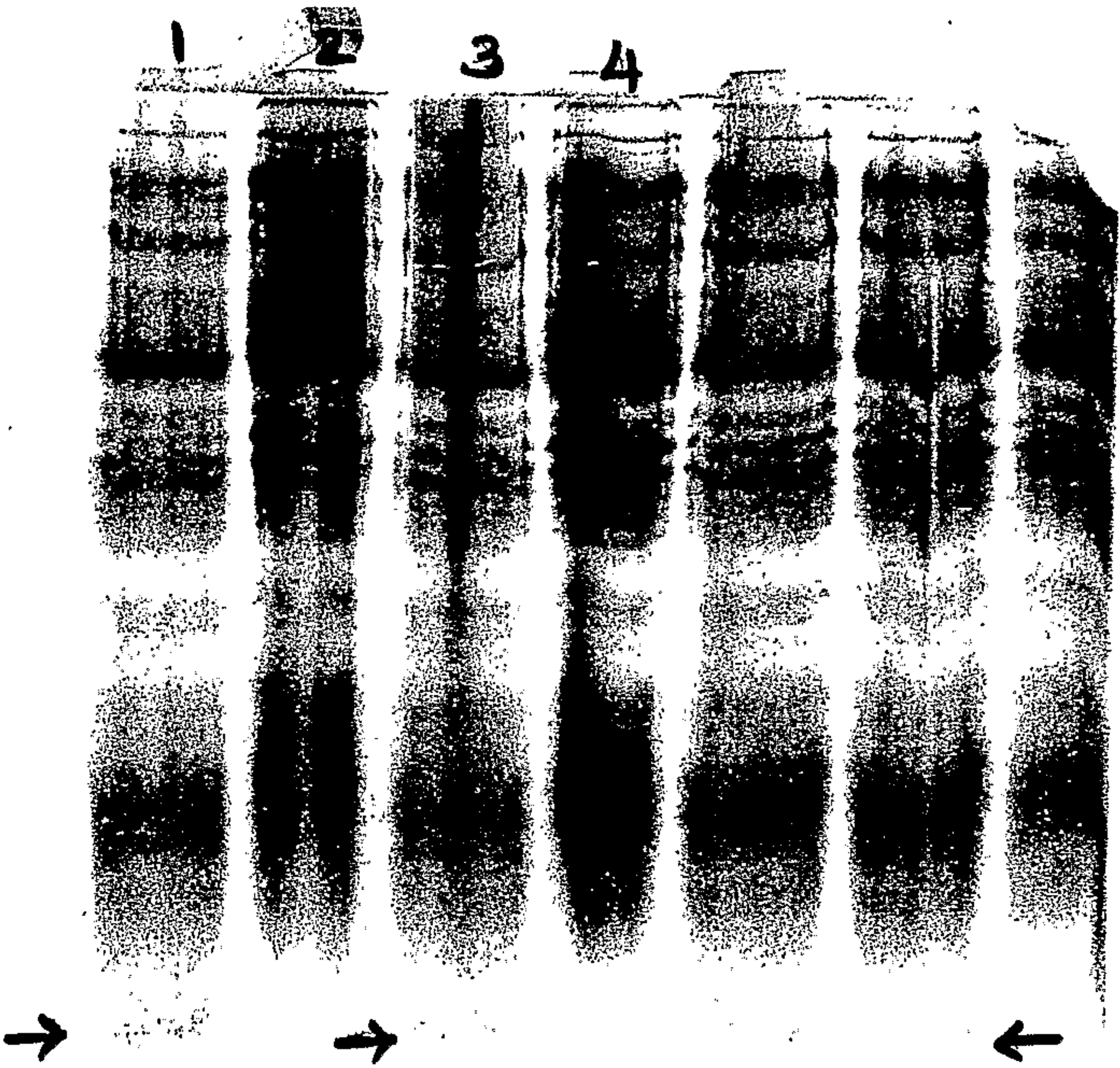




FIG. 32

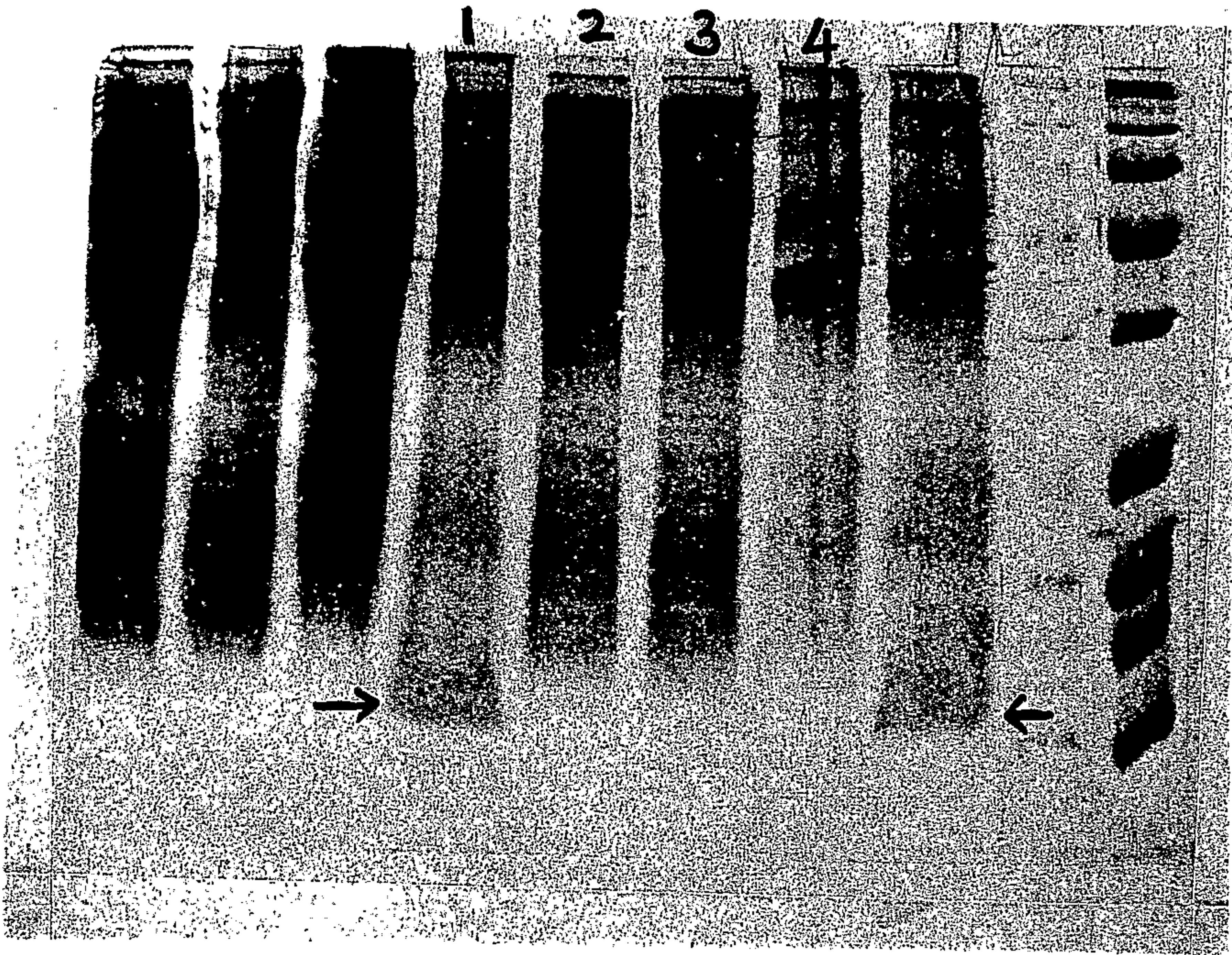


FIG. 33

Fig 33. Polyclonal antibody titers to Recombinantly expressed Multivalent Protein and the peptides

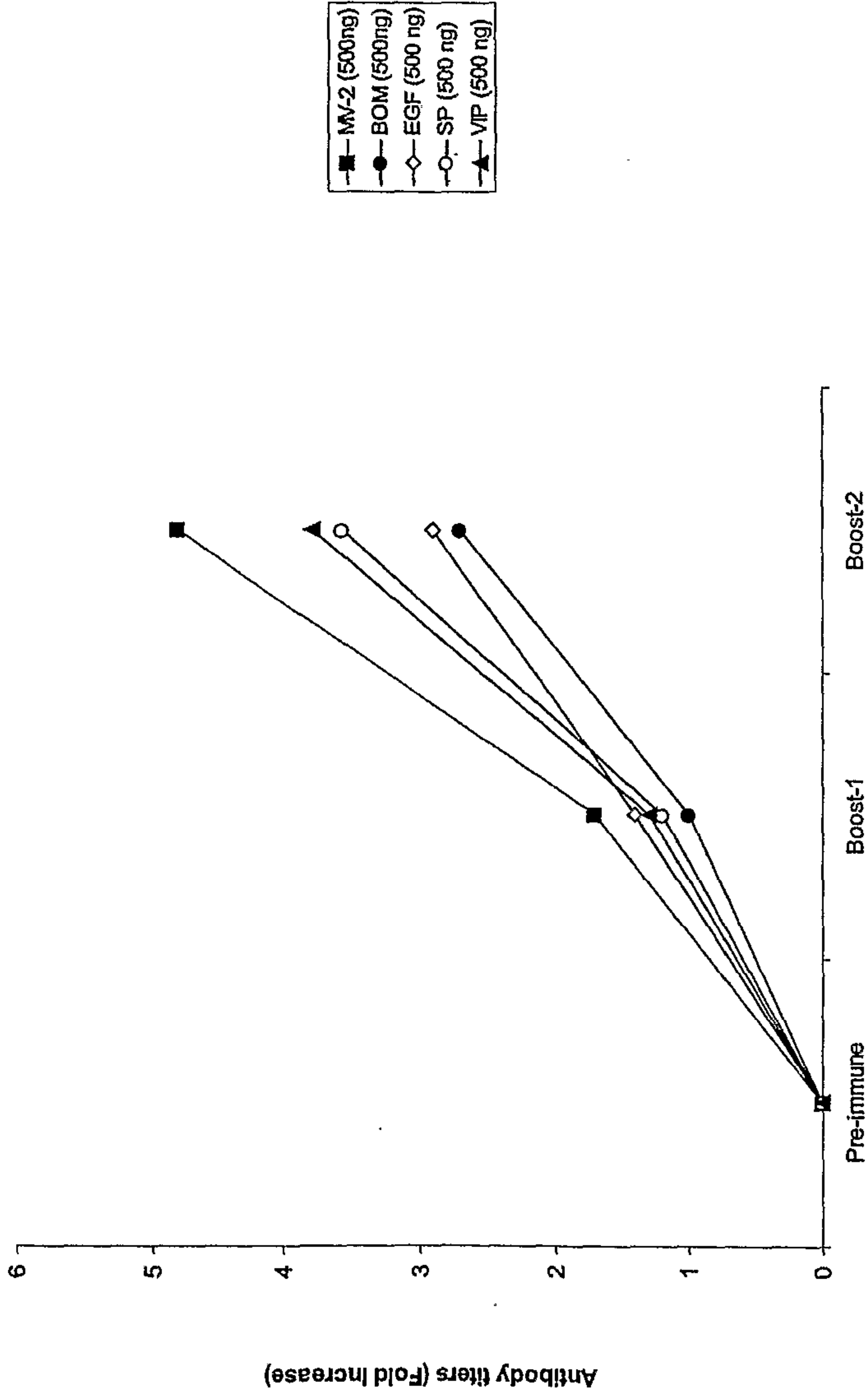




FIG. 34

Fig 34 Immunoblot of tumor lysates with polyclonal antibodies

