ISSN 0101-3084

CNEN/SP

ipen Instituto de Pesquisas Energéticas e Nucleares

MONOCLONAL ANTIBODY AS RADIOPHARMACEUTICAL

Iracelia Torres de Toledo e SOUZA, Helena OKADA, Constância Pagano Gonçalves da SILVA

IPEN-Pub-339

JUNHO/1991

SÃO PAULO

IPEN-Pub-339 JUNHO/1991

MONOCLONAL ANTIBODY AS RADIOPHARMACEUTICAL

Iracelia Torres de Toledo e SOUZA, Helena OKADA, Constância Pagano Gonçalves da SILVA

DEPARTAMENTO DE PROCESSAMENTO

CNEN/SP
INSTITUTO DE PESQUISAS ENERGÉTICAS E NUCLEARES
SÃO PAULO - BRASIL

Série PUBLICAÇÃO IPEN

INIS Categories and Descriptors

B13.30

MONOCLONAL ANTIBODIES
RADIOPHARMACEUTICALS
IMMUNOLOGY
ELECTROPHORESIS
SCINTISCANNING

IPEN - Doc - 3999

Aprovado para publicação em 16/05/91.

Nota: A redação, ortografia, conceitos e revisão final são de responsabilidade do(s) autor(es).

MONOCLONAL ANTIBODY AS RADIOPHARMACEUTICAL*

Iracelia Torres de Toledo e SOUZA, Helena OKADA
Constância Pagano Gonçalves da SILVA

COMISSÃO NACIONAL DE ENERGIA NUCLEAR-SP
INSTITUTO DE PESQUISAS ENERGÉTICAS E NUCLEARES
Caixa Postal 11049 - Pinheiros
05499 - São Paulo - BRAZIL

ABSTRACT

The purification of anti-CEA monoclonal antibody 4C11 belonging to IgG_{2a} subclass from mouse ascitis, donated by Ludwig Institute, Brazil was developed. The fragmentation of purified IgG_{2a} by pepsin digestion and analytical studies by polyacrilamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) were done as preliminary assessment for their specific application in immunoscintigraphy.

(*) Paper presented at the 4th European Symposium on Radiopharmacy and Radiopharmaceuticals, in Baden-Switzerland, May 1-4, 1991.

ANTICORPO MONOCLONAL COMO RADIOFÁRMACO *

Iracelia Torres de Toledo e SOUZA, Helena OKADA
Constância Pagano Gonçalves da Silva

COMISSÃO NACIONAL DE ENERGIA NUCLEAR-SP
INSTITUTO DE PESQUISAS ENERGÉTICAS E NUCLEARES
Caixa Postal 11049 - Pinheiros
05499 - São Paulo - BRASIL

RESUMO

A partir do fluido ascítico de camundongo, contendo o anticorpo monoclonal anti-CEA 4C11, subclasse IgG_{2a}, doado pelo Instituto Ludwig/Brasil, desenvolveu-se procedimentos imunoquímicos objetivando a: separação da imunoglobulina IgG_{2a}, clivagem pepsínica da IgG_{2a} para obtenção de fragmentos bivalentes e monitoração por eletroforese de gel de poliacrilamida na presença de dodecil sulfato de sódio, como uma metodologia preliminar indispensável ao alcance de um reagente imunológico adequado ao uso clínico.

(*) Trabalho apresentado no 4th European Symposium on Radiopharmacy and Radiopharmaceuticals, Baden-Suiça, de 1º a 4 de maio de 1991.

INTRODUCTION

Since of the development of Hybridoma Technology (Köhler and Milstein,1975) monoclonal antibodies have found an increasing number of applications and uses. The availability of monoclonal antibodies to tumour associated antigens having a high degree of specificity has contributed to more extensive **in vivo** use of radiopharmaceuticals. It was soon obvious that, prior the clinical applications, a lot of immunochemical work has to be done for each monoclonal antibody. It was necessary to develop affinity chromatography methods in order to isolate the specific IgG fraction (Primus et al.,1977). The intact IgG molecule is the easiest to use but because of its size and its ability to bind to Fc receptors, it may not be an ideal radiopharmaceutical. Fragments of the IgG molecule lacking the Fc portion is the most appropriate to use. Reduction of the immunoglobulin (IgG) size to fragments by enzymatic cleavage has been extensively described in the literature (Lamoyi,1986). The use of fragments has resulted in markedly distinct blood clearance and tumour localization patterns from that intact immunoglobulin (Larson et al.,1983).Classicaly papain and pepsin have been the enzymes employed in the generation of immunoreactive fragments of monoclonal antibodies (Nisonoff,1960, 1961). The purity of immunoglobulin IgG and their fragments are conveniently monitored by polyacylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The SDS-PAGE is a powerful tool for protein separation and characterization. Several systems for SDS-PAGE have been described, the most commonly is that devised by Laemmli,1970 using disc gel electrophoresis which was later adapted to slab gels by Studier,1973. The system is characterized by a discontinuity in the buffer pH and in the polyacrylamide pore size. The gel is divided into lower **running** gel and a shorter upper **stacking** gel.

The aim of this study was to develop an **experimental protocol** to optimize the purification of anti-CEA monoclonal antibody 4C1' belonging to IgG_{2a} subclass, fragmentation of the purified immunoglobulin IgG_{2a} subclass and analytical studies by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) as a preliminary assessment for their specific application in immunoscintigraphy.

MATERIAL AND METHODS

Ascitic fluid from BALB/c mouse carrying the anti-CEA 4C11 monoclonal antibody IgG_{2a} subclass was generously provided by Ludwig Institute, São Paulo/Brazil.

1. PURIFICATION OF ANTI-CEA 4C11 IgG_{2a}

Anti-CEA 4C11 IgG_{2a} monoclonal antibody is purified by affinity chromatography Protein A-Sepharose according to Ey et al., 1978.

PROCEDURE

- 1.5g of Protein A-Sepharose (Pharmacia Fine Chemicals) is resuspended in 5.0 ml 10mM phosphate buffered saline (PBS) pH 8.0 and packed into a small column (e.g. 10 ml glass-pippete).
- The column is "pre-cycled" prior to use with 0.1 M sodium citrate-citric acid pH 3.0 to remove bound material.
- The column is equilibrated with 0.1M phosphate pH 8.0.
- Sample (1 ml of ascitic fluid is mixed with 0.5 ml of 0.1 M phosphate buffer pH 8.0)
- Sample at pH 8.0 is added to the column; in these conditions all IgG are retained and all other proteins are eluted.
- The column is washed with 0.1M phosphate pH 8.0.
- The column efluent is monitored at 280nm an LKB UVICORD S.flow monitor and recorder.
- IgG_{2a} is eluted from the column using sodium citrate-acid citric pH 4.5.
- Denaturation of IgG_{2a} is minimized by adding sufficient 1M Tris-HCl pH 9.0 for each tube to ensure neutralization of the collected fractions.
- Fractions of approximately 3.0 ml are collected of flow rate of 0.46 ml/min.
- After each use the column is washed with 0.1M sodium citrate-citric acid pH 3.0 to eliminate found material, reequilibrated at pH 8.0 and stored at $4^{o}C$.
- The concentration of IgG_{2a} is determined by optical density at 280nm using an extinction coefficient $\varepsilon^{1\%} = 14$.
- The purity of IgG_{2a} is evaluated by SDS-PAGE.

2. FRAGMENTATION OF MONOCLONAL ANTIBODY

The optimization of the different parameters, such as enzyme/IgG_{2a} ratio, incubation time, temperature and buffer pH, is essential for the success of the fragmentation process (Lamoyi,1983,1986).

SOLUTIONS

- Sodium acetate 0.1M pH 7.0
- Acetic acid 2M
- Sodium acetate buffer 0.1M pH 4.2
- Tris-HCl buffer 2M pH 8.0
- Sodium hidroxide 0.5N
- Pepsin (2mg/ml) sodium acetate buffer 0.1M pH 4.2

PROCEDURE

- Immediately before digestion, adjust the pH of the purified IgG_{2a} to 4.2 by adding 2M acetic acid, and warm the solution to 37°C.
- Add 1 mg of pepsin per 20 mg of IgG_{2a}.
- Incubate the mixture at 37°C for 24 hours.
- to stop the digestion add 1/40 volume of 2M Tris-HCl pH 8.0 and raise the pH to 8.0 with 0.5N NaOH.
- $F(ab')_2$ fragments are separated from intact antibody and Fc portions by application to a Protein A-Sepharose column equilibrated in phosphate buffer 0.1M pH 8.0. The $F(ab')_2$ fragments are eluted with void volume, while undigested IgG_{2a} are eluted after equilibration of the column with citrate-citric acid buffer pH 4.5.
- The optical density at 280nm is used to determine protein concentrations, using the extinction coefficient $\epsilon^{1\%} = 14$ for fragments as well as whole antibody (Dernignot et al.,1989).
- The purity of the final material is evaluated by SDS-PAGE.

3. SDS-ELECTROPHORESIS

The basis 30:8/100 acrylamide N,N methylethylene diamine-bis acrylamide formulation of Shapiro et al.,1967 is used throughout at either 5%, 8% or 10% final acrylamide concentration, depending upon the desired molecular weight range.

Acrylamide poisoning: acrylamide is a neurotrotoxin (Kuperman, 1958), it must never be pipetted by mouth, or allowed to contact the

skin. Always wear mask and gloves when weighting the power.

SLAB GEL ELECTROPHORESIS APPARATUS

The apparatus for molding gels is available commercially but it is excessively expensive. Fortunately a slab gel suited for the slab gel electrophoresis can be made simply and inexpensively from acrilic plastic by a machine shop.

Essentially the apparatus consists of:

- Slab gel electrophoresis tanks: two buffer reservoir (lower and upper).
- A pair of glass plates (14cmx16cmx0.3cm). One of the plates presents 2 cm deep and 13.2 cm long notch cut out from one of the edges;
- Three acrylic spacers (1 cm wide and 1 mm thick). The common method of assembly is to use three spacers one of each side and one for the base of glass plate assembly. These are sealed by dripping molten 3% agar in water around the edges after assembly.
- The slab "running" gel is prepared between two glass plates. The plates are clamped together with six large binder clips.
- The spacer along the botton edge is removed after the polyacrylamide gel has polymerized and prior to electrophoresis.
- One acrilic plastic comb 10 wells (1 mm). The comb normally to mold small slots into the "stacking" gel for sample application.
- Microlitre syringes; for loading samples onto the gel.
- Pipettors with plastic disposable pipette tips.
- Power supply.
- Water pump.

REAGENTS AND SOLUTIONS

Details of buffer and stock solutions are given in TABLE 1.

PROCEDURE

- Assemble apparatus.
- "Running" gel. Selected desired acrylamide concentration (TABLE 2).
- "Stacking" gel (TABLE 3).
- Load sample: sample to be run on SDS slab gels should be prepared in SDS sample buffer (O'Farrel,1975) (TABLE 4).

TABLE 1

SOLUTIONS FOR SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS
(Double distilled water should be used throughout)

SOLUTION	RECIPE
SOLUTION A 30% acrylamide-bis-acrylamide (1:36)	30g acrylamide,8g N,N'methylene-bis-acrylamide in water to 100ml.Store at 4°C protected from light.
SOLUTION B Lower gel buffer	to 80ml water,add 18.2g Tris and 0.4g SDS. Titrate to pH 8.8 with 6N HCl,then make up 100ml. Store at 4°C.
SOLUTION C 30% acrylamide-bis-acrylamide (1:16)	30g acrylamide,1,6g N,N'methlene-bis-acrylamide in water to 100ml.Store at 4°C protected from light.
SOLUTION D Upper gel buffer	to 80ml water,add 6.06g Tris and 0.4g SDS. Titrate to pH 6.8 with 6N HCl,then make up to 100ml. Store at 4°C.
SDS 20% Sodium dodecyl sulfate	to 100ml water,add 20g SDS.The solution should be both clear and colourless.It is stable at room temperature for several weeks but precipitates in the cold.
10% ammonium persulphate	to 1ml water,add 20mg ammonium persulphate.This solution is unstable and should be made fresh just before use.
N,N,N',N'-tetramethylethylene TEMED	TEMED used as supplied.It is stable in undiluted solution at 4°C in a dark bottle.
Stock electrode buffer solution (electrode buffer)	to 900ml water,add 24g Tris and 115.2 glycine. Titrate to pH 8.5 with 6N HCl,then make up to 100ml. Store at 4°C. (4x stock,0.1% SDS when diluted)
Sample solubilizing buffer (SDS-Sample	to 18.5ml water,mix 2.5ml solution D;2.5ml 20% SDS;2.0ml glycerol;0.1ml EDTA (0.1M in water). Store small aliquots frozen.
Molecular weight markers	A number of proteins standards are commercially available.The sample should be prepared according to the manufacturer specifications.
Fixing solution	50%(v/v) methanol;10%(v/v) acetic acid;40% water.
Staining solution	0.2%(w/v) Coomassie Blue in the fixing solution.
Destaining solution	10%(v/v) methanol;7%(v/v) acetic acid;83% water.
Bromophenol Blue	0.1%(w/v) water.

TABLE 2

RUNNING GELS

The recipes tabulated below yield sufficient volume of gel mixture for one standard-size slab gel

% Acrylamide	5.0	8.0	10.0
Doubly distilled water (ml)	11.7	9.7	8.4
Solution A (ml)	3.3	5.3	6.7
Solution B (ml)	5.0	5.0	5.0
Ammonium persulphate (ml)	0.3	0.3	0.3

Solutions are mixed and deaerated in a water pump.

TEMED (µl)	20.0	20.0	20.0

Immediately after addition of TEMED the running gel is rapidly poured between the glass plates, up to about 2.5cm below the notch. The gel is then gently overlayed with double distilled water. In the ammonium persulphate TEMED system, TEMED catalyses the formation of free radicals from persulphate and these in turn initiate polymerization.

TABLE 3

STACKING GEL

The recipes tabulated below yield sufficient volume of gel mixture to polymeryzed on top the "running gel".

Double distilled water (ml)	3.17
Solution C (ml)	0.50
Solution D (ml)	1.25
Ammonium Persulphate (ml)	0.075

Solutions are mixed and deaerated in a water pump.

TEMED (µl)	20

-Remove the overlay on to the top of the gel. Insert the comb and fill the empty space with the stacking gel solution. Polymerization will occur within 30 min.
-After polymerization the comb is carefully removed to expose the sample wells.
-Note that the acrylamide concentration of the stacking gel for the SDS discontinuous system is constant irrespective of the acrylamide concentration chosen for the running gel.

TABLE 4

PREPARATION OF THE SAMPLES

Samples and standards are diluted in electrode buffer and are mixed with an adequate volume of the mixture tabulated below.

MIXTURE	
SDS sample buffer (µl)	370
Beta-mercaptoethanol (µl) (reduced ngent)	10
Bromophenol Blue (µl)	20

- Each sample should be heated at 100°C for 2-3 min.
- **Sample size:** The maximum sample volume possible is determined by the size of the sample wells that is formed in the stacking gel.
- Tipically, load 20-40 µl sample, containing 2-20 µg protein. SDS sample buffer can be prepared without beta-mercaptoethanol if proteins are to be run without reducing disulfide bonds. Beta-mercaptoethanol is used to disrupt polypeptide disulfide bonds.

- Load gels intoslab gel tanks with the notched front plate facing the upper reservoir.
- Fill the lower reservoir with electrode buffer.
- Remove any bubbles trapped between the glass gel plates at botton of the gel with a syringe and bent needle.
- Fill the top reservoir with electrode buffer.
- Run at 100V (constant current) at room temperature until the bromophenol, blue line reaches the botton of the gel (4-5 hours). The anode is the lower electrode and the cathode is the upper one.
- Turn off current, remove gel and discard the "stacking" gel (save the SDS electrode buffer for use in the lower reservoir during the next run).
- Place slab gels into stain (0.2% Coomassie blue) for 1 hour. Pour off the stain and save.
- Destain with destaining solution.

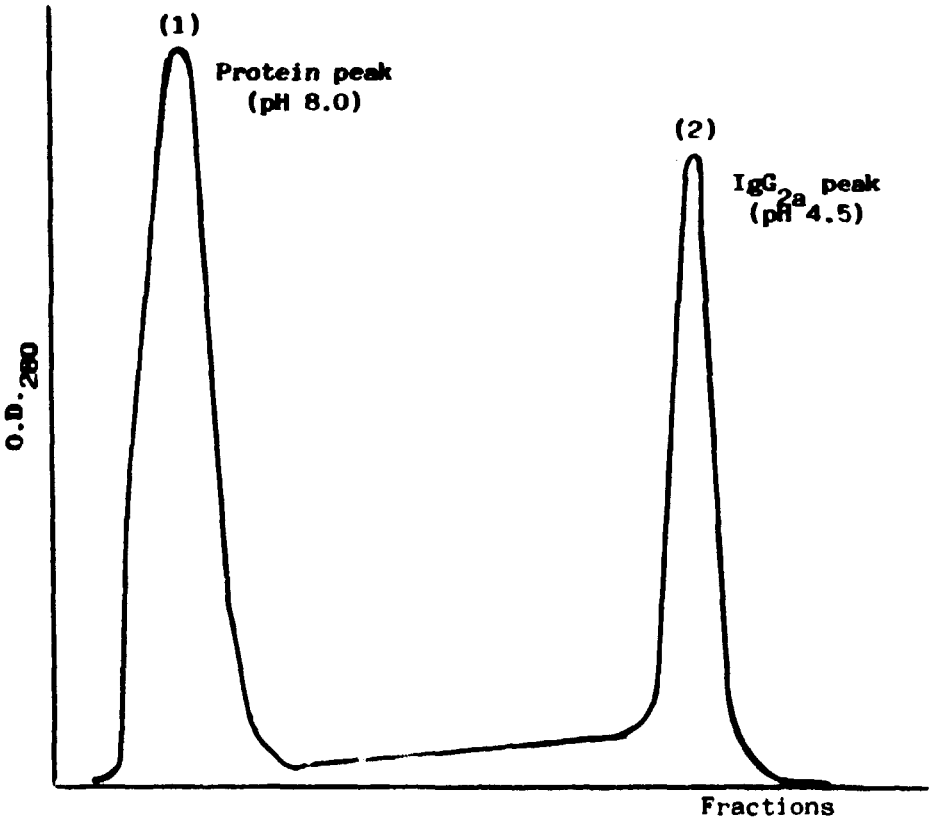

Fig 1. Elution of IgG_{2a} from Protein A-Sepharose (ascitic fluid 1ml was mixed with buffer pH 8.0). Fractions eluted at pH 4.5 were collected.

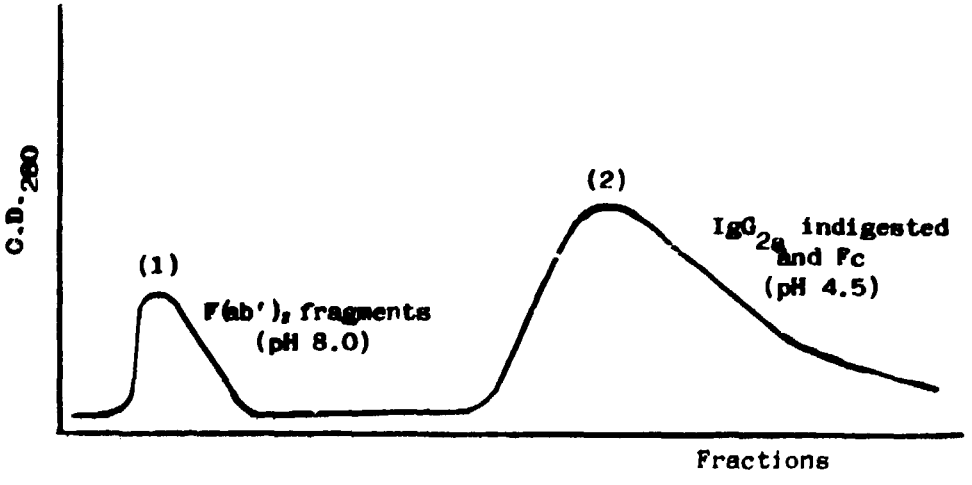

Fig 2. Elution of $F(ab')_2$ fragments from Protein A-Sepharose. The $F(ab')_2$ fragments were eluted with void volume at pH 8.0 while indigested IgG_{2a} and Fc were eluted after equilibration of the column at pH 4.5.

RESULTS

1. PURIFICATION OF ANTI-CEA 4C11 IgG_{2a}

Ascite containing 4C11 monoclonal antibody IgG_{2a} class was adjusted to pH 8.0 and passaged through the Protein A-Sepharose column which was washed sequentially at pH 8.0 and pH 4.5. At pH 4.5 amounts of IgG_{2a} were collected in 4-5-6 effluent fractions. The optical density at 280nm was used to determine IgG_{2a} concentrations, using the extinction coefficient $\epsilon^{1\%}_{1cm} = 14$. At a concentration of 1.4 mg/ml; the OD_{280} of the fractions was: 3.09; 3.10 and 3.00; representing a IgG_{2a} concentration of 2.20; 2.20 and 2.14 mg/ml respectively. The fraction size was 3.0 ml.

The OD_{280} profile of this elution is depicted in **Figure 1.**

2. FRAGMENTATION OF MONOCLONAL ANTIBODY

The bivalent $F(ab')_2$ fragments were prepared by incubating the purified IgG_{2a} with pepsin at pH 4.2 for 24 hours at 37°C. The protein to enzyme ratio was 20:1.

The $F(ab')_2$ fragments were separated from IgG_{2a} and proteolytic fragments by application to a Protein A-Sepharose column equilibrated at pH 8.0. The $F(ab')_2$ fragments appeared in the unbound fractions. Its concentration was estimated from OD_{280} as well as whole IgG_{2a}: at a concentration of 1.4 mg/ml, the OD_{280} of the fractions was: 0.228; 0.302; 0.222 representing a $F(ab')_2$ fragments concentration of 0.163; 0.215 and 0.159 mg/ml respectively. Fraction size was 3.0 ml.

The OD_{280} profile of this elution is depicted in **Figure 2.**

The final yield from purified IgG_{2a} to purified $F(ab')_2$ fragments (fraction 5) was approximately 10% of the starting material.

3. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The purity of IgG_{2a} and their $F(ab')_2$ fragments were monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). The SDS-PAGE was carried out in 10% gels.

The purified IgG_{2a} and $F(ab')_2$ fragments were shown to be homogeneous with SDS-PAGE.

REFERENCES

1. DEMIGNOT, S.; GARNETT, M.C.; BALDWIN, R.W. Mouse IgG_{2b} monoclonal antibody fragmentation: preparation and fragmentation of Fab, Fc and Fab/c fragments. *J. Immunol. Methods. 121:*209-17, 1989.
2. EY, P.L.; PROWSE, S.J.; JENKIN, C.R. Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using Protein A-Sepharose. *Immunochemistry. 15:*429-36, 1978.
3. KOHLER, G.; MILSTEIN, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature. 256:*495-5, 1975.
4. KUPERMAN, A.S. Effects of acrylamide on the central nervous system of the rat. *J. Pharmacol. Exp. Therap. 123:*180-92, 1958.
5. LAEMMILI, U.K. Cleavage of structural proteins during the assembly of the bead of bacteriophage. *Nature (London). 227:*680-4, 1970.
6. LAMOYI, E.; NISONOFF, A. Preparation of $F(ab')_2$ fragments from mouse IgG of various subclasses. *J. Immunol. Methods. 56:*235-243, 1983.
7. LAMOYI, E. Preparation of $F(ab')_2$ fragments from mouse of various subclasses. In: LANGONE, J.J.; VAN VUNAKIS, H. eds. *Methods in enzymology.* New York,N.Y., Academic Press, 1986. v.121,p.652-63.
8. LARSON, S.M.; CARRASQUILLO, J.A.; KROHN, K.A. Localization of ^{131}I labelled p97-specific Fab fragments in human melanoma as a basis of radiotherapy. *J. Clin. Invest. 72:*2101-14, 1983.
9. NISONOFF, A.; WISSLER, F.C.; WOERNLEY, D.L. Properties of univalent fragments of rabbit antibody isolated by specific absoption.*Arch. Biochem. 88:*241-55, 1960.
10. NISONOFF, A.; MARKUS, G.; WISSLER, F.C. Separation of univalent fragments of rabbit antibody by reduction of a single labile disulphide bond. *Nature. 189:*293-5, 1961.
11. PRIMUS, F.J.; MAC DONALD, R.; GOLDENBERG, D.M.; HANSEN, H.J. Localization of GW-39 human tumours in hamsters by affinity-purified antibody to carcinoembryonic antigen. *Canc. Res. 37:*1544-7, 1977.

12. SHAPIRO, A.L.; VINUELA, E.; MAIZEL, J. Molecular weight estimation of polypeptide chains by electrophoresis in SDS polyacrylamide gels. *Biochem. Biophs. Commun. 28:*815-20, 1967.

13. STUDIER, F.W. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol. 79:*237-48, 1973.