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(54) **Titre : DERIVES D'ANTICORPS HUMANISE QUI RECONNAIT LES ANTIGENES Y-6 ET B-7-2 DES GROUPES
SANGUINS DIFUCOSYL LEWIS**
(54) **Title: HUMANIZED ANTIBODY DERIVATIVES THAT RECOGNIZE DIFUCOSYL LEWIS BLOOD GROUP
ANTIGENS Y-6 AND B-7-2**

(57) **Abrégé/Abstract:**

Human/mouse chimeric and humanized monoclonal antibodies recognizing the difucosyl Lewis blood group antigens Y-6 and B-7-2 are disclosed. They can be used in the treatment of cancer of epithelial origin, of small cell lung cancer and of HIV-infections, especially of AIDS.



A B S T R A C T:

Human/mouse chimeric and humanized monoclonal antibodies recognizing the difucosyl Lewis blood group antigens Y-6 and B-7-2 are disclosed.

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HUMANIZED ANTIBODY DERIVATIVES THAT RECOGNIZE DIFUCOSYL LEWIS BLOOD GROUP ANTIGENS Y-6 AND B-7-2

The use of monoclonal antibodies (= Mabs) in therapeutic applications is gaining increasing acceptance. One such group of Mabs, of murine origin, is BR55-2 and fragments thereof having the same specificity and their variants, disclosed in e.g. Wistar EP 285 059, M. Blaszyk-Thurin et al, J. Biol. Chem. **262** (1987)/372-379, or Z. Steplewsky et al, Hybridoma **9**(1990) 201-210. These publications also disclose their preparation and their use in the detection and therapy of, basically, cancer of epithelial origin. The BR55-2 class of antibodies recognizes the difucosyl Lewis blood group antigens Y-6 and B-7-2 normally associated with cancer of epithelial origin. Mabs with specificity of BR55-2 are also useful for immunotherapy of HIV-infections, since the Lewis Y antigen is also selectively expressed on HIV infected cells.

There are, however, several drawbacks in using murine Mabs for therapeutic purposes in humans. First, such antibodies can induce a human anti-mouse antibody response; second, the half life of murine Mabs in the circulation is relatively short compared to human immunoglobulin; third, the Fc portion of murine Mabs may not elicit ADCC or CDC as effectively as the Fc portion of a human antibody. To overcome these possible problems recombinant DNA technologies have been applied to develop Mabs in which a part of the original mouse components are substituted by analogous human components. One such approach are human/mouse chimeric Mabs, containing the variable region of the murine antibody and the constant region of human immunoglobulin heavy and light chains. A further development in this direction consists in the construction of "fully humanized" antibodies by recombinant DNA technology in which only the minimum necessary parts of the parent mouse antibody, the complementarity determining regions (CDRs), are combined with human variable region frameworks and human constant regions. For the design and construction of these "fully humanized" Mabs, sequence homology and molecular modelling may be used to select a combination of mouse and human sequence elements that would further reduce immunogenicity while retaining the binding properties.

One embodiment of this invention concerns human/mouse chimeric Mabs recognizing the difucosyl Lewis blood group antigens Y-6 and B-7-2. A further embodiment of this invention concerns "fully humanized" monoclonal antibodies recognizing the difucosyl Lewis blood group antigens Y-6 and B-7-2. More particularly it concerns monoclonal antibodies containing only the minimum necessary parts of the parent mouse antibody BR55-2. It concerns also processes for the production of these antibodies and their use as pharmaceuticals.

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In one aspect, the invention provides a humanized monoclonal antibody that recognizes difucosyl Lewis blood group antigens Y-6 and B-7-2 comprising a humanized light chain variable region, a human light chain constant region, a humanized heavy chain variable region, and a human heavy chain constant region, wherein the humanized light chain variable region has a sequence according to SEQ ID NO:15; and the humanized heavy chain variable region has a sequence according to SEQ ID NO:13 or the SEQ ID NO:14.

A first step of the development of these new Mabs, the cloning and sequencing of the heavy chain and light chain variable domain cDNA for the murine Mab IgG3 BR55-2, can be carried out in the following manner:

The variable domain cDNA for the heavy chain and light chain of murine Mab BR55-2 was cloned by the anchored PCR method (Chiang Y.L., Sheng-Dong R., Brown A. and Larrick J.W.: Bio Techniques 7, 360-366 [1989]), which is outlined in Figure 1. First, a total RNA preparation was prepared using the hot phenol method. Briefly, 1×10^7 Mab BR55-2 hybridoma cells (ATCC HB9324) were — — — — — resuspended in 1.2 ml of RNA extraction buffer (50 mM sodium acetate, pH 5.2, 1% SDS), vortexed and incubated with 0.6 ml of phenol, pH 5.2, at 65° C for 15 minutes, followed by another 15 minutes incubation on ice. The extract was spun in a microfuge; the aqueous phase was recovered and ethanol precipitated twice. The RNA pellet was resuspended in water and quantitated at OD₂₆₀. cDNA was synthesized from the total RNA using reverse transcriptase (5 µg total RNA, 40 ng dT₁₂₋₁₈ (Pharmacia), 200 units of M-MLV reverse transcriptase (BRL), 40 units of RNasin (Pomega), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl₂ and 0.5 mM each dNTP in a 20 µl reaction volume). The G-tailing was achieved with terminal deoxynucleotidyl transferase (TdT) (cDNA, 15 units TdT (BRL), 0.1 M potassium cacodylate, pH 7.2, 2 mM CoCl₂, 0.2 mM DTT and 1 mM dGTP in a 20 µl reaction volume). Under the conditions described, tails generally contained about 20 bases. One half of the G-tailed product was then amplified to generate the V_L gene and the other half amplified to provide the V_H gene using Taq polymerase. The V_L gene is amplified with the primer mc45 (sequence shown in Figure 2a), that anneals to the G tail, and a primer mc46 (Figure 2b) that anneals to the constant region of the kappa light chain. The V_H gene was amplified with primers mc45 (Figure 2a) and mc47 (Figure 2c) that anneals to the constant region of gamma chains. EcoRI and HindIII sites are included in the upstream and downstream primers for convenient subcloning into pUC18 vector. An alternate set of restriction sites (XbaI and SacI) are also included in the primers for the rare event that EcoRI and HindIII sites are present in the variable region genes. The PCR reactions were performed in a programmable heating block using 30 rounds of temperature cycling (92° C for 1 minute, 50° C for 2 minutes and 72° C for 3 minutes). The reaction included the G-tailed product, 1 µg of each primer and 2.5 units of Taq polymerase (Perkin Elmer Cetus) in a final volume of 100 µl, with the reaction buffer recommended by the manufacturer. The PCR product bands were excised from a low-melting agarose gel, digested with restriction enzymes and cloned into pUC18 vector for sequence determination. The nucleotide sequence and the translated amino acid sequence of the light chain and heavy chain variable domain is shown in Figures 3 and 4. The initiation codon is underlined. The first amino

acid of the mature protein is marked 1. Complementarity determining regions (CDRs) are underlined and labeled.

The generation of the human/mouse chimeric Mabs can be carried out in the following manner:

1. Construction of expression vectors

Separate expression vectors were used to express the chimeric human BR55-2 IgG1 and IgG3 antibody light and heavy chains: pVk for the light chain, pVg1 for the gamma 1 heavy chain and pVg3 for the gamma 3 heavy chain. Diagrams of these vectors, with relevant restriction sites indicated, are presented in Figures 5 to 7. First pVk and then pVg1 and pVg3 are described in detail below; nucleotide position numbers start with 1 at the EcoRI site and refer to the complete plasmids.

Plasmid pVk. Proceeding clockwise (Figure 5), pVk first contains the human cytomegalovirus (CMV) major immediate early (IE) enhancer and promoter (Boshart M. et al., Cell **41**, 521-520 [1985]). The function of the promoter is to initiate transcription of the light chain gene at nucleotide 536, and the function of the enhancer, extending over approximately nucleotides 12 to 418, is to strongly increase the level of transcription (Boshart M. et al., Cell **41**, 521-520 [1985]). Thus the part of human CMV used is regulatory; no proteins are encoded. The CMV region is preceded by a short oligonucleotide linker used to connect it to the EcoRI site of the preceding pBR322 fragment.

The CMV region is followed by another linker containing an XbaI site. The variable region of an antibody light chain gene such as BR55-2 may be cloned into the XbaI site. The XbaI site is followed by part of a genomic clone (Hieter P.A. et al.: Cell **22**, 197-207 [1985]) of the human kappa light chain constant region (C_L), including the coding sequence, polyadenylation (poly A) signal, and part of the preceding intron.

The C_L region is followed by a gene encoding xanthine guanine phosphoribosyl transferase (gpt), together with regulatory elements (enhancer, promoter, splice signals, poly A signal) from Simian Virus 40 (SV40) needed for transcription. The function of this region, which was taken as a unit from the plasmid pSV2-gpt (Mulligan R.C. & Berg P.: Proc. Natl. Acad. Sci. USA **78**, 2072-2076 [1981]), is to provide a selectable drug-resistance marker after transfection of pVk into mammalian cells. Moving counter-clockwise within this one unit, first there is an SV40 segment containing the SV40 enhancer and early promoter (Reddy V.B. et al.: Science **200**, 494-502 [1978]), to ensure strong transcription initiation. This segment is followed by the coding sequence of the E. coli gpt gene (Richardson K.K. et al.: Nucleic Acids Research **11**, 8809-8816 [1983]). The gpt gene is followed by an SV40 segment containing the small t antigen intron, believed to increase mRNA levels, and then another SV40 segment containing a

poly A signal for ending the mRNA transcript. The direction of transcription of the gpt gene is opposite to that of the kappa light chain gene.

Finally, pVκ contains a large part of the widely used E. coli vector plasmid pBR322 (Sutcliffe J.G.: Cold Spring Harbor Symp. Quant. Biol. 43, 77-90 [1979]), comprising the origin of replication and ampicillin resistance gene (amp), respectively used for growth and selection in E. coli. These procaryotic elements are expected to be non-functional after pVκ is transfected into mammalian cells.

Plasmid pVg1^c. This plasmid is similar to pVκ but contains a heavy chain instead of light chain constant region and a different selectable marker. Specifically, proceeding clockwise (Figure 6), pVg1 contains the same CMV enhancer and promoter for strong transcription initiation as pVκ, inserted with the same EcoRI and XbaI linkers. The variable region of an antibody heavy chain such as BR55-2 can be inserted at the XbaI site. That site is followed by part of a genomic clone (Ellison J.W. et al.: Nucleic Acids Research 10, 4071-4079 [1979]), containing the human gamma 1 heavy chain constant region (C_H) including the C_H1, hinge (H), C_H2 and C_H3 exons with the intervening introns, part of the intron preceding C_H1, and a poly A site following C_H3.

The C_H region is followed by a gene encoding hygromycin B phosphotransferase (hyg), together with regulatory elements (enhancer, promoter, splice signals, poly A signal) from SV40 needed for transcription. This unit is identical to the gpt unit in pVκ, except that hyg replaces gpt. The hyg gene (Gritz L. & Davies J.: Gene 25, 179-188 [1983]) was cloned from an E. coli plasmid and confers resistance to the antibiotic hygromycin B, so it can be used as a selectable marker after transfection into mammalian cells. Finally, pVg1 contains the same part of the plasmid pBR322 as pVκ, containing the origin of replication and amp gene for use in E. coli.

Plasmid pVg3^c. This plasmid is identical to pVg1^c except that the XbaI-BamHI fragment containing the human gamma 1 heavy chain constant region is replaced by a XbaI-BamHI fragment containing the human gamma 3 heavy chain constant region (Figure 7), including the C_H1, hinge (H), C_H2 and C_H3 exons with the intervening introns, part of the intron preceding C_H1, and a poly A site following C_H3 (Takahashi N. et al.: Cell 29, 671-679 [1982]). The gamma 3 hinge region differs from the gamma 1 hinge region in that the former is comprised of 4 exons separated by 3 introns.

Both pVκ, pVg1^c and pVg3^c were constructed from their component parts in a number of steps by standard methods (Sambrook J. et al.: Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, N.Y. [1989]), including synthesis of oligonucleotide linkers. Their structure was carefully verified during and after construction by restriction mapping and sequencing.

2. Construction of variable domain segments

Chimeric BR55-2 light chain. The actual expressed chimeric BR55-2 light chain gene consists of two adjacent parts: a human genomic kappa constant region built into the vector pVk (see above), and the murine light chain variable region (V_L) constructed by PCR. To generate the V_L XbaI fragment, primers were constructed to anneal to the 5' and 3' ends of the murine cDNA clone. The 5' primer was constructed to include an XbaI site and a consensus CCACC sequence followed by the first ATG codon and 15 nucleotides of the signal peptide sequence. The 3' primer included the last 15 nucleotides of the variable region gene, followed by 23 nucleotides which are the same as the sequence that follows J_{K4} in the mouse genomic sequence. The primer also includes an XbaI site. The PCR generated fragment was then digested with XbaI and cloned into the XbaI site in the pVk vector. Thus, the cloned segment (Figure 8) encodes the V_L domain, including the J segment and a typical immunoglobulin leader (signal) peptide, which is cleaved off as the light chain is secreted. In addition, the segment includes the same 23 base pairs after the J segment that follow the mouse J_{K4} segment. The purpose of these nucleotides is to provide a splice donor signal to ensure that the intron between the V_L region and the downstream C_L region (Figure 5) is correctly spliced out. The correct orientation and sequence of the complete variable region (V_L) segment in pVk was then verified by sequencing again. All manipulations were done by standard methods (Sambrook J. et al.: Molecular Cloning, A Laboratory Manual, 2nd ed, Cold Spring Harbor Laboratory Press, N.Y. [1989]). Thus, the complete chimeric BR55-2 light chain gene consists of 1926 bp between an XbaI and a BamHI site (Figure 5). It contains a variable region exon (including leader and J segments), followed by a short intron and then a constant region exon. The particular kappa constant region used is of the Inv3 allotype (Hleter P.A. et al.: Cell 22: 197-207 [1985]), which occurs in 80% of the Caucasian population and 70% of the Black population (Sell S.: Immunology, Immunopathology and Immunity, 3rd ed. Harper & Row, Hagerstown, pp. 28 [1980]). The DNA following the termination codon of the C_L segment contains a presumptive poly A signal (Boshart M. et al.: Cell 41, 521-520 [1985]) to allow termination of the mRNA transcript.

Chimeric BR55-2 heavy chain. The actual expressed heavy chain gene consists of two adjacent parts: a human genomic gamma 1 (or gamma 3) constant region built into the vector pVg1^c (or pVg3^c), and the murine heavy chain variable region (V_H), constructed by PCR in the same manner as described above and cloned into the XbaI site of pVg1^c (or pVg3^c) (Figures 6 and 7). The PCR generated segment (Figure 9) encodes the V_H domain, including the J segment and a typical immunoglobulin leader (signal) peptide, which is cleaved off as the heavy chain is secreted. In addition, the segment includes the same 19 base pairs after the J segment that follow the mouse J_{H3} segment. The purpose of these nucleotides is to provide a

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splice donor signal to ensure that the intron between the V_H and the downstream C_H1 is correctly spliced out. The orientation and sequence of the complete segment was verified after cloning into the XbaI site of the pVg1^c (or pVg3^c).

Thus, the complete chimeric BR55-2 heavy chain gene contains a variable region exon (including leader and J segments), followed by a short intron and then the constant region (Figures 6 and 7). The gamma 1 constant region was obtained as a human genomic clone and therefore itself consists of 4 exons – C_H1 , H (hinge), C_H2 and C_H3 – separated by 3 introns. The gamma 3 constant region is similar to the gamma 1 constant region except that an extended H (hinge) is composed of four exons separated by three introns. The particular gamma 1 constant region used has the Gm(1,17) allotypic markers (Ellison J.W. et al.: Nucleic Acids Research 10, 4071–4079 [1979]), which occur in 60% of the Caucasian population and 100% of the Black population (Sell S.: Immunology, Immunopathology and Immunity, 3rd ed. Harper & Row, Hagerstown, pp. 28 [1980]). The DNA following the termination codon of the C_H3 segment contains a presumptive poly A signal (Ellison J.W. et al.: Nucleic Acids Research 10, 4071–4079 [1979]) to allow termination of the mRNA transcript.

3. Transfected cell line

Host cell system. The host cell line was Sp2/0–Ag14 (ATCC CRL 1581), which was developed by M. Shulman, C.D. Wilde and G. Kohler in 1978 (Shulman M. et al.: Nature 276, 269–270 [1978]). They isolated it as a re-clone of Sp2/HL–Ag, which was derived from Sp2/HLGK, a hybrid between a BALB/c spleen cell with antisheep red blood cell activity and the mouse myeloma line P3x63Ag8. Sp2/0–Ag14 does not survive in HAT medium and has the important characteristic that it does not synthesize or secrete any immunoglobulin chains. For this reason, the cell line is commonly used as a fusion partner in generating hybridomas. It is equally suitable as a host cell line for producing a chimeric antibody, because only the transfected immunoglobulin genes will be expressed.

A vial of Sp2/0–Ag14 cells obtained from the American Type Culture Collection was thawed and then passaged several times to produce enough cells to perform DNA transfections. The cells were grown and the transfectants maintained in DMEM medium + 10% fetal bovine serum (FBS).

Transfection of cells. Transfection was by electroporation using a Gene PulserTM apparatus (Bio–Rad) at 360 V and 25 μ FD capacitance according to the manufacturer's instructions. Before transfection, the light chain– and heavy chain–containing plasmids were linearized using BamHI, extracted with phenol–chloroform, and ethanol–precipitated. All transfections were done using 20 μ g plasmid DNA and about 10^7 cells in PBS. The cells from each transfection were plated into one 96–well tissue culture plate. After 48 hours, selective medium was

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applied.

Cells were selected in DMEM + 10% FBS + HT media supplement (Sigma) + 1 µg/ml mycophenolic acid. After the wells had become confluent with surviving colonies of cells, medium from each well was assayed for the presence and quantity of secreted antibodies by ELISA. A high-yielding clone from each transfection was grown up to produce antibody for purification.

4. Purification of chimeric BR55-2 antibodies.

IgG1 chimeric antibody. BR55-2 IgG1 chimeric antibody was purified from serum-free conditioned media plus 0.5% FBS using Protein A Sepharose chromatography. 4 liter of culture medium was concentrated 16-fold using a Pellicon system equipped with a 10,000 MW CO cellulose membrane. The pH of the concentrate was adjusted to 8.5 using 1 M Tris and the slight precipitate that formed was removed by centrifugation. The concentrate was then loaded, at a flow rate of 2 ml/min, onto a 1.6 x 12 cm Protein A SepharoseTM column (Pharmacia) which was pre-equilibrated with 0.15 M NaCl, 50 mM Tris, pH 8.5 until the absorbance at 280 nm returned to baseline, and the bound IgG1 was eluted with 0.15 M NaCl, 0.1 M acetic acid. The fractions were collected into one-tenth volume of sodium bicarbonate, to neutralize the pH, and the pooled fractions were dialyzed against PBS and filter sterilized. Protein concentration was estimated by taking OD at 280 nm (1 mg/ml = 1.35 OD). Antibodies were more than 95% pure based on SDS-PAGE analysis and size-exclusion HPLC (see Figure 42).

IgG3 chimeric antibody. BR55-2 IgG3 chimeric antibody was purified from serum-free conditioned media using Protein G agarose chromatography. 4 liter of culture medium were concentrated 16 fold using a Pellicon system equipped with a 10,000 MW CO cellulose membrane. The pH of the concentrate was adjusted to 5.0 using 1M acetic acid and the slight precipitate that formed was removed by centrifugation. The concentrate was then loaded, at a flow rate of 1 ml/min, onto a 1 x 6.5 cm Protein G agarose column (Pierce) which was pre-equilibrated with 0.15 M NaCl, 20 mM sodium acetate, pH 5.0. The column was washed with 0.15 M NaCl, 20 mM sodium acetate, pH 5.0 until the absorbance at 280 nm returned to baseline and the bound IgG3 was eluted with 0.1 M glycine/HCl, pH 2.8. The fractions were collected into one-tenth volume of sodium bicarbonate, to neutralize the pH, and the pooled fractions were dialyzed against PBS and filter sterilized. Protein concentration was estimated by taking OD at 280 nm (1 mg/ml = 1.35 OD). Antibodies were more than 95% pure based on SDS-PAGE analysis and size-exclusion HPLC (see Figure 42).

The "fully humanized" Mabs can be generated in the following manner:

1. Construction of expression vectors

Separate expression vectors were used to express the humanized BR55-2 IgG1 antibody light and heavy chains: pVk for the light chain, pVg1 for the gamma 1 heavy chain. Diagrams of

these vectors, with relevant restriction sites indicated, are presented in Figures 5 and 10. pV_k is as described above, pVg₁ is described in detail below; nucleotide position numbers start with 1 at the EcoRI site and refer to the complete plasmids.

Plasmid pVg₁. This plasmid is similar to pV_k but contains a heavy chain instead of light chain constant region and a different selectable marker. Specifically, proceeding clockwise (Figure 10), pVg₁ contains the same CMV enhancer and promoter for strong transcription initiation as pV_k, inserted with the same EcoRI and XbaI linkers. The variable region of an antibody heavy chain such as humanized BR55-2 can be inserted at the XbaI site. That site is followed by part of a genomic clone (Ellison J.W. et al., Nucleic Acids Research **10**, 4071-4079 [1982]) containing the human gamma 1 heavy chain constant region (C_H) including the C_H1, hinge (H), C_H2 and C_H3 exons with the intervening introns, part of the intron preceding C_H1, and a poly A site following C_H3.

The C_H region is followed by a gene encoding a mutant gene for dihydrofolate reductase (dhfr), together with regulatory elements (enhancer, promoter, splice signals, poly A signal) from SV40 needed for transcription. This unit is identical to the gpt unit in pV_k, except that dhfr replaces gpt. The mutant dhfr gene (Simonsen C.C. et al., Proc. Natl. Acad. Sci. USA **80**, 2495-2499 [1983]) confers resistance to methotrexate, so it can be used as a selectable marker after transfection into mammalian cells. The mutant dhfr was cloned from a wild-type gene with a single amino acid substitution at position 22 (Leu to Arg) and can be employed as a dominant selectable marker in cultured cells expressing normal levels of wild-type dihydrofolate reductase. This marker also allows to select higher antibody producers by subjecting cells to increased level of methotrexate. Finally, pVg₁ contains the same part of the plasmid pBR322 as pV_k, containing the origin of replication and amp gene for use in E.coli.

Plasmids pVg₂, pVg₃ and pVg₄. These plasmids were constructed to express the human γ ₂, γ ₃ and γ ₄ heavy chains, respectively. The plasmids are identical to pVg₁ except that the XbaI-BamHI fragment containing the human γ ₁ heavy chain constant region is replaced by XbaI-BamHI fragments containing the γ ₂, γ ₃ and γ ₄ heavy chain constant regions, respectively (Takahashi N., et al., Cell **29**, 671-679 [1982] and Ellison, J. et al., Proc. Natl. Acad. Sci. USA **79**, 1984-1988 [1982]). All the plasmids were constructed from their component parts in a number of steps by standard methods (Sambrook J. et al.: Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, N.Y.[1989]), including synthesis of oligonucleotide linkers. Their structure was carefully verified during and after construction by restriction mapping and sequencing.

2. Computer modeling of humanized variable region domain

In order to retain high binding affinity in the humanized antibody, the general procedures of Queen et al. (Queen, C. et al., Proc.Natl.Acad.Sci. USA, 86: 10029-10033 (1989)) were followed. The more homologous a human antibody is to the original murine antibody, the less likely will combining the murine CDRs with the human framework be to introduce distortions into the CDRs that could reduce affinity. The first step in the designing of humanized antibody is to perform a sequence homology search to select the best framework. Comparison of variable regions of BR55-2, murine IgG3, with a few selected human antibodies is shown below (E.A. Kabat et al., Sequences of Proteins of Immunological Interest, 4th Edition [1987], U.S. Dept. of Health and Human Services) (sequence homology including CDRs given in percentage):

Ab	VL	VH
Eu	54%	43%
Siø	57%	41%
Ou	48%	41%
Lay	56%	64%
Pom	56%	64%
Tew	77%	-

Pom was selected to provide the framework for the humanized heavy chain and Tew for the humanized light chain variable region. Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce the possibility of incompatibility in the assembling of the two chains. The BR55-2, murine IgG3, light chain variable region, however, shows a significantly higher homology to the Tew framework compared to any others.

Therefore, Tew was chosen to provide the framework for the humanized light chain variable region, despite the absence of available sequence for the heavy chain. Pom was chosen to provide the framework for the heavy chain because of its high homology to the BR55-2, murine IgG3, heavy chain sequence.

Next, the computer programs ABMOD and ENCAD (Zilber, B.T. et al., Biochemistry 29: 10032-10041) were used to construct a molecular model of the BR55-2, murine IgG3, variable domain. Inspection of the refined model of murine BR55-2 revealed several amino acid residues in the framework that have significant contacts with the CDR residues (category 4 below). To design the humanized light and heavy chain BR55-2 variable regions, at each position the amino acid was chosen to be the same as in the Tew or Pom sequence, respectively, unless that position fell in one or more of the four categories:

- (1) The position fell within a CDR.
- (2) The Pom or Tew amino acid was unusual for human antibodies at that position, whereas the BR55-2, murine IgG3, amino acid was typical for human antibodies at that position.
- (3) The position was immediately adjacent to a CDR.
- (4) The model described above suggested that the amino acid may be physically close to the antigen binding region (CDRs).

Category	Light Chain	Heavy Chain
1	24-39, 55-61, 94-102	31-35, 50-66, 99-108
2	108	82, 87
3		109
4	54	73, 74, 109

The humanized light chain and heavy chain sequences are labeled L-hu-BR55-2 and H-hu-BR55-2/1, respectively. Binding affinity measurements showed that the binding affinity of the humanized antibody is roughly four fold lower than that of the BR55-2 mouse/human chimeric IgG1 antibody.

Since the overall electrostatics of a protein can affect the binding of a substrate, this effect was investigated in an effort to increase the binding affinity of the humanized antibody. The amino acid sequences of the humanized heavy chain and the murine heavy chain were compared to identify framework residue differences that result in a charge change. Several humanized heavy chain variants with single or double amino acid substitutions from the murine sequence were constructed. One variant with substitutions at position 42 (Gly to Glu) and position 44 (Gly to Arg) increases the binding affinity by two fold. One variant with a substitution at position 95 (Tyr to His), which is involved in the interfacing with the light chain, also increases binding affinity by two fold. A variant chain, incorporating these three substitutions, the sequence of which is labeled H-hu-BR55-2/2, was constructed and shown to bind to the antigen with affinity within two fold of the BR55-2 mouse/human chimeric IgG1 antibody.

It was also found that substituting residue 75 in the heavy chain with the murine residue enhances antibody secretion. The humanized heavy chain sequence, which incorporates this additional change, is labeled H-hu-BR55-2/3. The heavy chains described above were then cotransfected each with the L-hu-BR55-2 light chain to produce the respective humanized antibodies.

The variants with the heavy chain sequence H-hu-BR55-2/2 and H-hu-BR55-2/3 were named BR55-2 humanized IgG1/2 and BR55-2 humanized IgG1/3 respectively. An alignment and comparison of the three humanized heavy chain variants is shown in Figure 11. A comparison of

the humanized light chain, L-hu-BR55-2, and the humanized heavy chain, H-hu-BR55-2/3, with the respective Tew and Pom sequences are shown in Figures 12 and 13.

3. Construction of variable domain segments

Humanized BR55-2 light chains. The actual expressed humanized BR55-2 light chain gene consists of two adjacent parts: a human genomic kappa constant region built into the vector pVκ (see above), and the humanized light chain variable region (V_L) constructed by total gene synthesis from oligonucleotides.

For the construction of light chain variable region gene, nucleotide sequences were selected that encode the protein sequences of the humanized light chain, including the signal peptide, generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences also included a splice donor signal from the $J_{\kappa 4}$ in the mouse genomic sequence and an XbaI site at each end. The gene was constructed from four overlapping synthetic oligonucleotides (Figure 14). For the variable domain gene, two pairs of overlapping synthetic oligonucleotides on alternating strands were synthesized that encompassed the entire coding sequences as well as the signal peptide and the splice donor signal. The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 base long with a 15 base overlap. Double stranded DNA fragments were synthesized with Klenow polymerase, digested with restriction enzymes, ligated to pUC18 vector and sequenced. The two fragments with the correct sequences were then ligated into the XbaI sites of pVκ expression vector.

Thus, the cloned segment encodes the humanized V_L domain, including the J segment and a typical immunoglobulin leader (signal) peptide, which is cleaved off as the light chain is secreted. In addition, the segment includes the same 23 base pairs after the J segment that follow the mouse $J_{\kappa 4}$ segment. The purpose of these nucleotides is to provide a splice donor signal to ensure that the intron between the V_L region and the downstream C_L region (Figure 5) is correctly spliced out. The correct orientation and sequence of the complete variable region (V_L) segment in pVκ was then verified by sequencing again. All manipulations were done by standard methods (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd ed., 1989).

Thus, the complete humanized BR55-2 light chain gene consists of the segment between a XbaI and a BamHI site (Figure 5). It contains a variable region exon (including leader and J segments), followed by a short intron and then a constant region exon. The particular kappa constant region used is of the Inv3 allotype (Hieter, P.A. et al., Cell 22: 197-207 (1980)), which occurs in 80% of the Caucasian population and 70% of the Black population (Sell, S., Immunol-

ogy, Immunopathology and Immunity, 3rd ed. (Harper and Row: Hagerstown, MD) pp.28 (1980)). The DNA following the termination codon of the C_L segment contains a presumptive poly A signal (Boshart, M. et al., Cell 41: 521-530 (1985)) to allow termination of the mRNA transcript.

Humanized BR55-2 heavy chains. The actual expressed heavy chain gene consists of two adjacent parts: a human genomic gamma 1 constant region built into the vector pVg1, and the humanized heavy chain variable region (V_H), constructed by total gene synthesis in the same manner as described above and cloned into the XbaI site of pVg1 (Figure 10). The XbaI fragment, which can be synthesized from four oligonucleotides (Figure 15), encodes the humanized V_H domain, including the J segment and a typical immunoglobulin leader (signal) peptide, which is cleaved off as the heavy chain is secreted. In addition, the segment includes the same 19 base pairs after the J segment that follows the mouse J_H3 segment. The purpose of these nucleotides is to provide a splice donor signal to ensure that the intron between the C_H and the downstream C_H1 is correctly spliced out. The orientation and sequence of the complete segment was verified after cloning into the XbaI site of the pVg1.

Thus, the complete humanized BR55-2 γ 1 heavy chain gene contains a variable region exon (including leader and J segments), followed by a short intron and then the constant region (Figs.6). The gamma 1 constant region was obtained as a human genomic clone and therefore itself consists of 4 exons - C_H1, H (hinge), C_H2 and C_H3 - separated by 3 introns. The particular gamma 1 constant region used has the Gm (Chiang, Y.L., Sheng-Dong, R., Brow, A. and Larrick, J.W., BioTechniques 7, 360-366 (1989)) allotypic markers (Ellison, J.W. et al., Nucleic Acids Research 10: 4071-4079 (1982)), which occur in 60% of the Caucasian population and 100% of the Black population (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory Press, NY) (1989)). The DNA following the termination codon of the C_H3 segment contains a presumptive poly A signal to allow termination of the mRNA transcript.

For expression of humanized BR55-2 IgG1, IgG2, IgG3 and IgG4, the XbaI fragment coding the humanized BR55-2 heavy chain variable region, including the signal sequence and the 3' splicing signal, was inserted into the XbaI site of the respective vectors. Orientation and sequence of the variable region gene was confirmed by restriction digestion and sequencing. Each of the heavy chain expressing plasmids was cotransfected with the humanized BR55-2 light chain expressing plasmid into SP2/0 cells.

4. Transfected cell lines

This step of the process is similar to the procedure described for the mouse/human chimeric Mabs. However, based on the methotrexate resistance introduced in the respective pVg1, pVg2, pVg3 and pVg4 it is possible to obtain a high yielding cell line by selection of antibody-producing cells from the transfection in 50 nM methotrexate. This can be done in the following manner.

Surviving cells were subjected to increasing concentrations of methotrexate (two fold step-wise) until the level of antibody production reaches the maximum. The best producing cells were then subcloned twice by limited dilution and the highest-yielding clone was selected for production of the respective antibody.

5. Purification of humanized Mabs

This step of the process can be carried out similarly to the procedure described for the mouse/human chimeric Mabs. The IgG1, IgG2 and IgG4 Mabs were purified using Protein A Sepharose columns, the IgG3 was purified using a Protein G Agarose column. Isotypes of the purified Mabs were confirmed by a human IgG subclass EIA kit (Isotypes, Inc. Newark, Delaware) (see also Figure 42).

The potential of an unconjugated antitumor Mab for tumor cell destruction is determined by its binding properties to the tumor associated antigen as well as by the constant domains responsible for activation of effector functions.

In general, the binding properties of a mouse/human chimeric Mab are similar compared to the binding properties of the parent murine Mab. However, fully humanized Mabs obtained by grafting of the CDR into human framework and reshaping by molecular modelling may exhibit lower binding affinity than the parent murine Mab and the mouse/human chimeric Mabs. Remarkably, the binding properties of the fully humanized Mabs described in this invention are still comparable to the binding properties of the mouse/human chimeric Mabs and the parent murine Mabs.

Murine Mabs BR55-2, depending on their subclass, activate both human complement and human effector cells for tumor cell destruction, the murine IgG3 subclass being most effective in this respect. In case of the mouse/human chimeric Mabs and the fully humanized Mabs with binding specificity of BR55-2 described in this invention the most active subclass for activation of human effector functions is human IgG1. However, the pattern of activation of human effector functions is different. While the complement activation ability is somewhat diminished in comparison to murine IgG3, the ability for activation of human effector cells for tumor cell destruction is superior.

The Lewis Y antigen is also selectively expressed on HIV-infected cells. Based on this observation Mabs with specificity of BR55-2 are also useful for immunotherapy of HIV-infection.

BR55-2 humanized IgG1/3 displays significant antiviral properties by reducing by more than 90% the infectivity of HIV-infected cultures of human PBMCs. Therefore BR55-2 mouse/human chimeric Mabs and BR55-2 humanized Mabs are promising for immunotherapy of HIV. Since the Lewis Y carbohydrate antigen is specified by the infected cell and not by the viral genome, HIV escape mutants (which are a major problem in therapy of AIDS) are highly unlikely to occur during immunotherapy with Mabs with specificity of BR55-2, leading to a unique advantage of such a therapy over existing treatment modalities.

The above mentioned properties and activities can be shown in the following tests and studies:

1. Binding of mouse/human chimeric and humanized variants of BR55-2 to SKBR5 breast cancer cell line

The Lewis Y carbohydrate antigen is strongly expressed on the surface of the human breast cancer cell line SKBR5. Both the BR55-2/ mouse/human chimeric Mabs and the BR55-2 humanized Mabs efficiently bind to this cell line in a cell-ELISA (see example 1 for experimental details and figures 16 to 18 for results). The binding of BR55-2 mouse/human chimeric IgG1 (bearing the same variable region as the parent murine IgG3 Mab) to this cell line was also compared with the binding of BR55-2 humanized IgG1/3 (bearing only the CDRs of the parent murine IgG3 Mab inserted in human framework) using fluorescence activated flow cytometry (see example 2 for experimental details). As shown in figures 19 and 20, the binding properties of both variants are comparable, the affinity is almost fully retained after the humanization procedure.

2. Complement dependent cytotoxicity mediated by mouse/human chimeric and humanized variants of BR55-2

The destruction of several Lewis Y antigen positive human tumor cell lines by BR55-2 mouse/human chimeric IgG1 and -IgG3 as well as by BR55-2 humanized IgG1/2 and -IgG1/3 via activation of human complement was tested in comparison to the parent murine IgG3 Mab. The cell lines used were: SKBR5: breast cancer; CATO: gastric cancer; MCF7: breast cancer; SW 948: colon cancer; SW2: small cell lung cancer. — — — — — — — — — —

The results are shown in Figures 21 to 30. They indicate that the — — — — — — — — — — human IgG3 subclass is significantly less active in complement mediated destruction than the human IgG1 subclass. The BR55-2 humanized IgG1 variants mediate tumor cell lysis comparable to the BR55-2 mouse/human chimeric IgG1. However, the parent mouse IgG3 Mab is more potent in complement dependent lysis (see example 3 for experimental details).

3. Antibody dependent cellular cytotoxicity mediated by mouse/human chimeric and humanized variants of BR55-2

The destruction of several Lewis Y antigen positive human tumor cell lines by BR55-2 mouse/human chimeric IgG1 and -IgG3 as well as by BR55-2 humanized IgG1/2 and -IgG1/3 via activation of human peripheral mononuclear cells as well as human monocytes and human granulocytes was tested in comparison to the parent murine IgG3 Mab. The cell lines used were: CATO: gastric cancer; — — — — — SKBR5: breast cancer; MCF7: breast cancer; SW948: colon cancer; SW2: small cell lung cancer. The human IgG3 subclass is less active in tumor cell destruction via activation of human effector cells than the human IgG1 subclass. However, the ADCC activity of BR55-2 mouse/human chimeric IgG1 as well as of the BR55-2 humanized IgG1 variants tested is significantly superior to the activity of the parent murine IgG3 Mab. This activity pattern is similar for human PBMCs, human monocytes and human granulocytes as effector cells. Interestingly, for BR55-2 humanized IgG1/2 in all experiments a higher efficacy was found than for BR55-2 humanized IgG1/3 which by itself is comparable to BR55-2 mouse/human chimeric IgG1 (see example 4 for experimental details). The results are shown in Figures 31 to 41.

4. Mixed cell HIV-Infectivity assay

Human PBMCs were infected with HIV and feeded after one week with fresh autologous serum and lymphocytes to provide complement and effector cells, respectively. After one week incubation with BR55-2 humanized IgG1/3 infectivity of the cell culture supernatant was titrated in MT-4 cells (see example 5 for experimental details). BR55-2 humanized IgG1/3 significantly inhibits the infectivity, especially in the presence of fresh serum. The results are shown in Table 1.

Table 1
Infectivity of supernatants of HIV-Infected human PBMC cultures treated with
BR55-2 humanized IgG1/3

	Titer* (heat inactivated serum)	Titer* (fresh serum)
BR55-2 humanized IgG1/3 (80 µg/ml)	1:316	1:100
PBS (control)	1:1000	1:3160

* end-point titers from titration of the PBMC supernatants in MT-4 cells.

The following examples illustrate the invention but are not limitative. All temperatures are given in degrees centigrade. The abbreviations have the following meanings:

ADCC:	antibody dependent cellular cytotoxicity
amp:	ampicillin
BSA:	bovine serum albumin
CDC:	complement dependent cytotoxicity
CDR:	complementarity determining regions
CMV:	cytomegalovirus
dhfr:	dihydrofolate reductase
DNA:	desoxyribonucleic acid
dGTP:	desoxyguanosine-5'-triphosphate
dNTP:	desoxynucleotide-5'-triphosphate
DTT:	dithiothreitol
EDTA:	ethylene diamine tetraacetic acid
ELISA:	enzyme-linked immunosorbent assay
FBS:	fetal bovine serum
Fc:	fragment crystallizable
FCS:	fetal calf serum
gpt:	guanine phosphoribosyl transferase
HAMA:	human anti-mouse antibody
HBSS:	Hank's buffered saline (Gibco)
HIV:	human immunodeficiency virus
Ig:	immunoglobulin
Mab:	monoclonal antibody
PAGE:	polyacrylamide gel electrophoresis
PBMC:	peripheral blood mononuclear cells
PBS def.:	phosphate buffered saline
PBS def.2:	PBS def. + 0.1 % EDTA + 0.1 % NaN ₃ + 1 % FCS (heat inactivated)
PCR:	polymerase chain reaction
PHA:	phytohemagglutinin
polyA:	polyadenylation
RNA:	ribonucleic acid
RPMI:	Roswell Park Memorial Institute
SCLC:	small cell lung cancer

SDS: sodium dodecyl sulfate
SV40: Simian Virus 40
TdT: terminal deoxynucleotidyl transferase

The materials referred to in the examples are as follows:

Cell lines: SKBR5: human breast cancer cell line
MCF7: human breast cancer cell line
SW948: human colon cancer cell line
CATO: human gastric cancer cell line
SW2: human small cell lung cancer line

Medium A: RPMI 1640 + 2 g/l NaHCO_3
100 U/ml Penicillin G
100 $\mu\text{g/ml}$ streptomycin sulfate
4 mM glutamine
10% FCS (heat-inactivated, γ -globulin-free)

Medium A2: Medium A without phenol red

HAT medium: hypoxanthine-aminopterin-thymidine

DMEM medium: Dulbecco's modified eagles medium

Lymphoprep: density 1.077 ± 0.001 g/ml

PBS complete: 138,0 mM NaCl
1,5 mM KOH
2,7 mM KCl
6,5 mM Na_2HPO_4
0,9 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
0,5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

PBS deficient: 138.0 mM NaCl
1.5 mM KOH
2.7 mM KCl
6.5 mM Na_2HPO_4
pH 7.2

Coating buffer: 15 mM Na_2CO_3
35 mM NaHCO_3
3 mM NaN_3
pH 9.6

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Staining buffer: 24.3 mM citric acid

51.4 mM Na_2HPO_4

pH 5.0

Washing buffer: 0.2% Triton X-100TM

2 % NaCl

in PBS deficient

Substrate solution: 40 mg o-phenylenediamine dihydrochloride

100 ml staining buffer

20 μl H_2O_2 (30 %)

$\text{Na}_2^{51}\text{CrO}_4$: 1 mCi/ml

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Example 1: Binding of BR55-2 mouse/human chimeric IgG1 and BR55-2 mouse/human chimeric IgG3 to SKBR5 cell line (cell-ELISA)

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with a suspension of SKBR5 cells to be tested at a concentration of 4×10^6 cells/ml (50 µl of cell suspension/well). After removal of the supernatant the cells are fixed with 50 µl glutardialdehyde (0.1 % in physiological saline) per well for 5 minutes at room temperature, the supernatant is removed, 200 µl/well of PBS def./1% BSA/0.1% NaN₃ are added and left for 1 hour at room temperature. After removal of the supernatants and washing twice with 200 µl PBS/Tween 20 (0.05%) per well, the antibody dilutions (100 µg/ml down to 0.08 µg/ml in PBS def.) are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl of ice-cold PBS/Tween 20TM (0.05%) per well and peroxidase-conjugated antibody is added. The conjugate used is goat anti-human IgG-peroxidase (such as the reagents of Chemicon Co.) 1:1000 in PBS def./2% FCS. After incubation for 45 minutes at 37° the wells are washed thrice with the above PBS/Tween 20 solution and then 100 µl of substrate solution is added to each well. After 5 minutes colour development is stopped by addition of 50 µl of 4N H₂SO₄/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (calibration is at 620 nm).

Example 2: Binding of BR55-2 mouse/human chimeric IgG1 and BR55-2 humanized IgG1/3 to SKBR5 cell line (fluorescence-activated flow cytometry)

SKBR5 cells are cultivated in medium A2, spun down at 400g, washed in PBS def. 2 and aliquoted into vials for fluorescence-activated flow cytometry (e.g. using FACScan; Becton Dickinson). To 10^6 cells in 500 µl medium A2 appropriate antibody dilutions are added in 250 µl PBS def. and incubated for 1 hour at 4°. After washing the cells with PBS def. 2 FITC labeled goat antihuman IgG is added (such as the reagents of Axell; 6 µg/vial in 100 µl PBS def.) and incubated overnight at 4°. The cells are washed as described above and resuspended in 300 µl PBS def. 2 (with 2 % paraformaldehyde). After 2 hours incubation at 4° 150 µl PBS def. 2 are added and the cell suspensions are analyzed using the flow cytometer.

Example 3: Complement dependent cytotoxicity (CDC) using human serum

On the day preceding the assay the respective tumor target cells are transferred into fresh medium A and kept at 37°/5% CO₂ in a cell culture flask.

⁵¹Cr labelling of the target cells:

The cells are collected from the culture flask and incubated at a concentration of 5×10^6 cells in 800 µl of medium A at 37°/5% CO₂ for 1 hour with 100 µCi Na₂⁵¹CrO₄. The cells are then

washed with medium A to remove the excess ^{51}Cr , resuspended in fresh medium A and their concentration is adjusted to 2.5×10^5 cells/ml.

CDC:

100 μl aliquots of this suspension of target cells are pipetted into each well and 50 μl aliquots of the antibody solution, diluted to the desired concentrations in PBS def., are added. Then 100 μl aliquots of a human serum (final dilution 1:2.5) are added per well and the cells are incubated overnight at $37^\circ/5\% \text{CO}_2$. The supernatants are harvested with a Skatron-Harvesting-Press and counted in a γ -counter. This yields the value for the experimental release. For determination of total ^{51}Cr release the cells are treated as above but with the human serum replaced by a solution of 2% SDS, 50 mM Na_2CO_3 and 10 mM EDTA. The value for spontaneous ^{51}Cr release is obtained by replacing the human serum with medium A and the antibody solution with 50 μl PBS def.

After counting the result is computed as follows:

$$\% \text{ lysis} = \frac{(\text{experimental release minus spontaneous release}) \times 100}{\text{total release minus spontaneous release}}$$

Example 4: Antibody-dependent cellular cytotoxicity (ADCC)

On the day preceding the assay the respective tumor target cells are transferred into fresh medium A and kept at $37^\circ/5\% \text{CO}_2$ in a cell culture flask.

^{51}Cr labelling of the target cells is effected as described in example 3.

Isolation of PBMC: 50 ml of heparinized fresh human blood are diluted with 50 ml of PBS complete containing 0.1% glucose. 15 ml aliquots of this solution are layered on top of 15 ml of Lymphoprep solution and the tubes are centrifuged at 800g for 30 minutes. The plasma supernatants are discarded, the PBMC layers are collected and diluted to 50 ml with PBS complete + 0.1% glucose. After centrifugation (250g, 10 minutes), resuspension of the pellet in 25 to 30 ml PBS complete + 0.1% glucose, and recentrifugation (350g, 10 minutes), the pellet is collected, suspended in medium A, the cells are counted and the suspension is diluted with medium A to about 2×10^6 to 9×10^6 cells/ml. 100 μl aliquots are pipetted into each well of a microtiter plate and the effector cells are incubated overnight at $37^\circ/5\% \text{CO}_2$.

Isolation of monocytes: Human monocytes are isolated via centrifugal elutriation as described (Thelen M. et al., Blood 75, 2223-2228 [1990]). The cells are resuspended in medium A and diluted to 10^7 cells/ml. 100 μl aliquots are pipetted into each well of a microtiterplate and incubated overnight at $37^\circ/5\% \text{CO}_2$.

Isolation of granulocytes: 50 ml of heparinized fresh human blood are diluted with 50 ml of PBS complete containing 0.1% glucose. 15 ml aliquots of this solution are layered on top of 15 ml of

Lymphoprep solution and the tubes are centrifuged at 800g for 30 minutes. The supernatants are discarded and the pellets are diluted in 30 ml HBSS. After gently rotating a further dilution is done to 1:5 with HBSS + 6 % Dextran. After 20 min (room temperature) erythrocytes are sedimented, the granulocytes in the supernatant are collected, spun down and washed twice with medium A. The cells are resuspended in medium A and diluted to 10^7 cells/ml. 100 μ l aliquots are pipetted into each well of a microtiterplate and incubated overnight at 37°/5% CO₂.

ADCC:

100 μ l of ⁵¹Cr-labelled target cells are added to the preincubated effector cells in the desired ratio of effector cells to target cells. 50 μ l of antibody solution diluted to the desired concentrations with PBS def. are added and the plate is incubated overnight (about 18 hours) at 37°/5% CO₂. The supernatants are then harvested with a Skatron-Harvesting Press and counted in a γ -counter. This yields the value for the experimental release.

Total ⁵¹Cr release is determined as above but replacing PBMC with 100 μ l of 2% SDS, 50 mM Na₂CO₃ and 10 mM EDTA and replacing the antibody solution with 50 μ l of PBS def. Spontaneous ⁵¹Cr release is obtained by replacing PBMC with 100 μ l of medium A and the antibody solution with 50 μ l of PBS def. The result is computed as described in example 3.

Example 5: Mixed cell HIV-infectivity assay

PBMC from a healthy donor were separated from peripheral blood and stimulated with 5 μ g/ml PHA for three days, inoculated with HIV_{III}B (M. Popovic et al., Science **224**, 497 [1984]) overnight, washed and cultured in growth medium. One week after inoculation when infection was indicated by syncytium formation, fresh serum and PBMCs were obtained from the same donor, and some of the serum was heat inactivated (56° for 30 min). Uninfected PBMC (0.6×10^6) were mixed with 0.3×10^6 infected PBMC and 300 μ l serum with or without heat inactivation. Appropriate concentration of BR55-2 humanized IgG1/3 or PBS was added to the mixture which was then cultured in a total volume of 1.5 ml growth medium per well of a 24-well cell culture plate. After one week a ten-fold dilution series of each supernatant was used for infection of MT-4 cells to get an approximate end point titer measured by production of HIV p24 antigen in culture supernatants of MT-4 cells as indicator for HIV-infections as described previously (J. Hansen et al., J.Virol. **65**, 6461 [1991]).

On view of the above experimental results chimeric and humanized Mabs of BR55-2 and fragments thereof having the same specificity and variants thereof, are thus indicated for use in the diagnosis and treatment of cancer of epithelial origin, e.g. breast-, colorectal-, ovarian-, prostate-, pancreatic- or gastric cancer, and of small cell lung cancer and for use in the treatment of HIV infections, especially of AIDS.

Since they show a restricted binding specificity associated with a lack of cross-reactivity to related antigens expressed on blood cells, e.g. erythrocytes, they are particularly suited for therapeutic use in humans.

For the above-mentioned use the dosage will, of course, vary depending upon e.g. the compound employed, the subject patient's age, the stage of disease, the mode of administration or the treatment desired, and can be determined by the specialist in each individual situation. It will also vary when the antibodies are used in combination with chemotherapeutic agents or immunostimulators. Administration is e.g. parenteral by injection or infusion. The dosage administered is e.g. of from about 10 mg to about 300 mg of chimeric or humanized Mab as defined above, given in intervals of 3 to 7 days, preferably by slow intravenous infusion.

The invention therefore also concerns a method of treatment of cancer of epithelial origin, e.g. breast-, colorectal-, ovarian-, prostate-, pancreatic- or gastric cancer, of small cell lung cancer and of HIV infections, especially of AIDS, which comprises administering to a subject in need of such treatment an effective amount of the antibody.

EXPLANATION OF THE FIGURES:

Figure 1: Immunoglobulin cloning strategy: V_H and V_L genes were cloned using the anchored polymerase chain reaction (PCR). cDNA was synthesized from 5 μ g of total RNA using reverse transcriptase and oligo dT as primers. A G-tail was attached to the 3' end of the cDNA using terminal deoxynucleotidyl transferase (TdT). The G-tail cDNA was then amplified using a pair of primers, one annealed to the constant region of light chain or heavy chain, the other annealed to the G-tail. Restriction sites are incorporated into the upstream and downstream primers for convenient cloning into pUC18 vector for sequence determination.

Figure 2: Primers: primers used in the anchored polymerase chain reactions.

- a) mc45 anneals to the G-tail;
- b) mc46 anneals to the constant region of kappa chain;
- c) mc47 anneals to the constant region of gamma chains. The sequence in parenthesis indicates a base degeneracy at the position. The degeneracy was introduced so that the primer would be able to recognize all classes of gamma chains.

Figure 3: BR55-2 murine IgG3 light chain^{variable domain}/sequence: the initiation codon is underlined. The first amino acid of the mature protein is marked 1. CDR's are underlined. 5' untranslated sequence is also given.

Figure 4: BR55-2 murine IgG3 heavy chain^{variable domain}/sequence: the initiation codon is underlined. The first amino acid of the mature protein is marked 1. CDR's are underlined. 5' untranslated sequence is also given.

Figure 5: Diagram of plasmid pVk: component parts are labeled, coding regions are shown as boxes, and restriction sites used in the construction are labeled.

Figure 6: Diagram of plasmid pVg1^c: component parts are labeled, coding regions are shown as boxes, and restriction sites used in the construction are labeled.

Figure 7: Diagram of plasmid pVg3^c: component parts are labeled, coding regions are shown as boxes, and restriction sites used in the construction are labeled.

Figure 8: BR55-2 light chain variable region (VL): the nucleotide sequence and the translated amino acid sequence of the variable region segment in pVk is shown. The XbaI sites are underlined. The splice donor signal is marked by arrow. The peptide signal is also translated. The first amino acid of the mature protein is labeled 1.

Figure 9: BR55-2 heavy chain variable region (VH): the nucleotide sequence and the translated amino acid sequence of the variable region segment in pVg1^c (or pVg3^c) is shown. The XbaI sites are underlined. The splice donor signal is marked by arrow. The peptide signal is also translated. The first amino acid of the mature protein is labeled 1.

Figure 10: Diagram of plasmid pVg1^h: coding regions are shown as boxes, component parts and restriction sites used in the construction are labeled.

Figure 11: Amino acid sequence of humanized BR55-2, heavy chain variants: amino acid sequence alignment of three humanized heavy chain variants. The top line shows the H-hu-BR55-2/1 sequence. Substitutions in H-hu-BR55-2/2 and H-hu-BR55-2/3 sequences are shown underneath. The CDR sequences are underlined.

Figure 12: Amino acid sequence of humanized BR55-2, light chain comparison with Tew sequence: amino acid sequence of the light chain of the humanized BR55-2 (upper line) compared with the Tew sequence (lower line). The three CDR's are underlined. Residues in the framework that have been replaced with mouse amino acids in the humanized antibody are double underlined.

Figure 13: Amino acid sequence of humanized BR55-2/3, heavy chain comparison with Pom sequence: amino acid sequence of the heavy chain of the humanized BR55-2/3 (upper line) compared with the Pom sequence (lower line). The three CDR's are underlined. Residues in the framework that have been replaced with mouse amino acids in the humanized antibody are double underlined.

Figure 14: Oligonucleotides: the four oligonucleotides to be used for the construction of the humanized BR55-2 light chain (L-hu-BR55-2 sequence).

Figure 15: Oligonucleotides: the four oligonucleotides to be used for the construction of the humanized BR55-2/3 heavy chain (H-hu-BR55-2/3 sequence).

Figure 16: Binding to SKBR5 breast cancer cell line (cell-ELISA):
 crosses = BR55-2 mouse/human chimeric IgG1;
 squares = BR55-2 mouse/human chimeric IgG3

Figure 17: Binding to SKBR5 breast cancer cell line (cell-ELISA):
 crosses = BR55-2 mouse/human chimeric IgG1;
 squares = BR55-2 humanized IgG1/2;
 losanges = BR55-2 humanized IgG1/3.

Figure 18: Binding to SKBR5 breast cancer cell line (cell-ELISA):
 crosses = BR55-2 humanized IgG2;
 dots = BR55-2 humanized IgG3;
 asterisks = BR55-2 humanized IgG4.

Figure 19: Binding to SKBR5 breast cancer cell line (fluorescence-activated flow cytometry):

- 1 = 60 µg BR55-2 mouse/human chimeric IgG1;
- 2 = 15 µg BR55-2 mouse/human chimeric IgG1;
- 3 = 3.75 µg BR55-2 mouse/human chimeric IgG1;
- 4 = 0.94 µg BR55-2 mouse/human chimeric IgG1;

Figure 20: Binding to SKBR5 breast cancer cell line (fluorescence-activated flow cytometry):

- 1 = 60 μ g BR55-2 humanized IgG3;
- 2 = 15 μ g BR55-2 humanized IgG3;
- 3 = 3.75 μ g BR55-2 humanized IgG3;
- 4 = 0.94 μ g BR55-2 humanized IgG3;

Figure 21: CDC to SKBR5 breast cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

- asterisks = BR55-2 mouse IgG3;
- crosses = BR55-2 mouse/human chimeric IgG1;
- squares = BR55-2 mouse/human chimeric IgG3.

Figure 22: CDC to SW948 colon cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

- asterisks = BR55-2 mouse IgG3;
- crosses = BR55-2 mouse/human chimeric IgG1;
- squares = BR55-2 mouse/human chimeric IgG3.

Figure 23: CDC to CATO gastric cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

- asterisks = BR55-2 mouse IgG3;
- crosses = BR55-2 mouse/human chimeric IgG1;
- squares = BR55-2 mouse/human chimeric IgG3.

Figure 24: CDC to SW2 small cell lung cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

- asterisks = BR55-2 mouse IgG3;
- crosses = BR55-2 mouse/human chimeric IgG1;
- squares = BR55-2 mouse/human chimeric IgG3.

Figure 25: CDC to SKBR5 breast cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 26: CDC to SKBR5 breast cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 27: CDC to MCF7 breast cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 28: CDC to SW948 colon cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 29: CDC to CATO gastric cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 30: CDC to SW2 small cell lung cancer cell line: complement-dependent cytotoxicity with human serum (1 : 2.5):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 31: ADCC to SKBR5 breast cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = .15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 mouse/human chimeric IgG3.

Figure 32: ADCC to SW 948 colon cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = 15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 mouse/human chimeric IgG3.

Figure 33: ADCC to CATO gastric cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = 15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 mouse/human chimeric IgG3.

Figure 34: ADCC to MCF 7 breast cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = 15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 mouse/human chimeric IgG3.

Figure 35: ADCC to SKBR5 breast cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = 15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 36: ADCC to SKBR5 breast cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = 15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 37: ADCC to MCF7 breast cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = 15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 38: ADCC to SW948 colon cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = 15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 39: ADCC to SW2 small cell lung cancer cell line: antibody dependent cellular cytotoxicity with human PBMC (E:T = 15:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 40: Human monocyte ADCC to SKBR5 breast cancer cell line: antibody dependent cellular cytotoxicity with human monocytes (E:T = 40:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3.

Figure 41: Human granulocyte ADCC to SKBR5 breast cancer cell line: antibody dependent cellular cytotoxicity with human granulocytes (E:T = 40:1):

asterisks = BR55-2 mouse IgG3;
crosses = BR55-2 mouse/human chimeric IgG1;
squares = BR55-2 humanized IgG1/2;
losanges = BR55-2 humanized IgG1/3;

Figure 42: Size-exclusion HPLC:

1 = BR55-2 mouse IgG3;
2 = BR55-2 mouse/human chimeric IgG1;
3 = BR55-2 humanized IgG1/2;
4 = BR55-2 humanized IgG1/3.

STARTING MATERIALS

Murine monoclonal antibodies BR55-2 are available from e.g. hybridomas BR55.2 (BR55-2/IgG3) and, respectively, BR55.2S2a (BR-55-2/IgG2a).

These hybridomas were originally deposited on February 17, 1987 and, respectively, March 10, 1987 with the American Type Culture Collection, Rockville, MD 20852, USA, under the provisions of the Budapest Treaty, under deposit numbers ATCC HB 9324 and, respectively, ATCC HB 9347.

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CLAIMS:

1. A humanized monoclonal antibody that recognizes difucosyl Lewis blood group antigens Y-6 and B-7-2 comprising a humanized light chain variable region, a human light chain constant region, a humanized heavy chain variable region, and a human heavy chain constant region, wherein the humanized light chain variable region has a sequence

D I V M T Q S P L S L P V T P G E P A S I S C R S S
 Q S I V H S N G N T Y L E W Y L Q K P G Q S P Q L
 L I S K V S N R F S G V P D R F S G S G S G T D F T
 L K I S R V E A E D V G V Y Y C F Q G S H V P F T F
 G Q G T K L E I K

and the humanized heavy chain variable region has a sequence

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F S D Y Y M Y W V R Q A
 P E K R L E W V A Y I S N G G G S S H Y V D S V K G R F T I S R D N S K N T L Y
 L Q M N S L R A E D T A L Y H C A R G M D Y G A W F A Y W G Q G T L V T V S S

or a sequence

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F S D Y Y M Y W V R Q A
 P E K R L E W V A Y I S N G G G S S H Y V D S V K G R F T I S R D N A K N T L Y
 L Q M N S L R A E D T A L Y H C A R G M D Y G A W F A Y W G Q G T L V T V S S.

2. The humanized monoclonal antibody of claim 1, wherein the human light chain constant region is a kappa chain constant region and the human heavy chain constant region is a gamma chain constant region.

3. The humanized monoclonal antibody of claim 2, wherein the gamma chain is a gamma 1 chain.

4. The humanized monoclonal antibody of claim 2, wherein the gamma chain is a gamma 4 chain.

5. A fragment of the antibody of any one of claims 1 to 4, wherein the fragment recognizes difucosyl Lewis blood group antigens Y-6 and B-7-2.

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6. A pharmaceutical composition comprising the antibody of any one of claims 1 to 4 or a fragment of claim 5 with a pharmaceutically acceptable carrier or diluent.

7. Use of an antibody of any one of claims 1 to 4 or a fragment of claim 5 as a pharmaceutical.

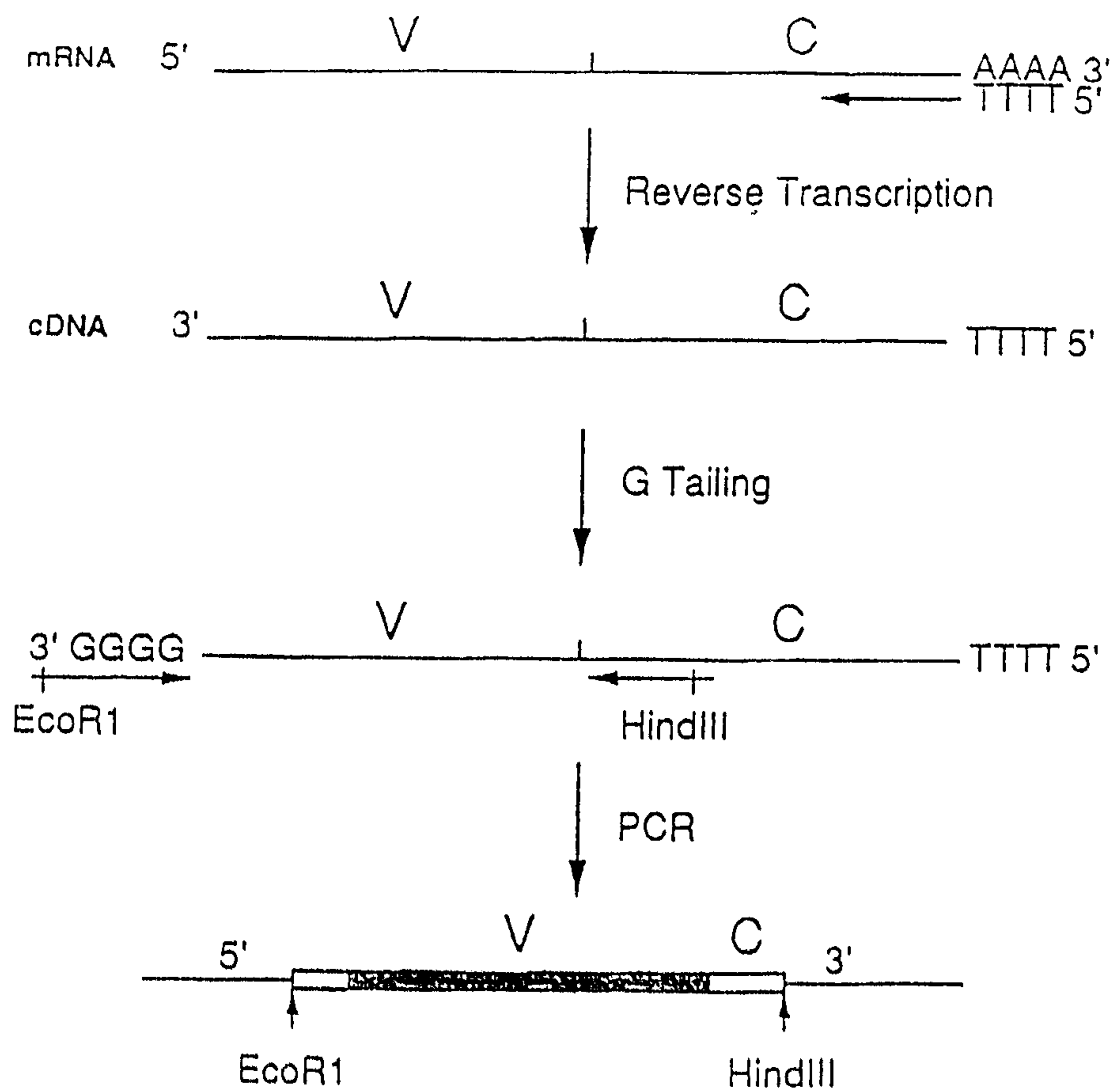
8. Use of a humanized monoclonal antibody of any one of claims 1 to 4 or a fragment of claim 5 in the manufacture of a medicament for the treatment or diagnosis of a cancer of epithelial origin.

9. Use of claim 8 wherein the cancer is gastric cancer, breast cancer, colon cancer or small cell lung carcinoma.

10. Use of a humanized monoclonal antibody of any one of claims 1 to 4 or a fragment of claim 5 in the manufacture of a medicament for the treatment of HIV infection.

Figure 1:

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Baker & Potvin*

Figure 2:

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- a) mc45 5' TATATCTAGAATTCCCCCCCCCCCCCCCCCCC 3'
- b) mc46 5' TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGCTGC 3'
- c) mc47 5' TATAGAGCTCAAGCTTCCAGTGGATAGAC(CAT)GATCGGG(GC)TGT(TC)GTTTGGC 3'

*Kirby, Lades, Gale,
Baker & Potvin*

Figure 3:

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10 20 30 40 50 60
ATCAGTCTCCTCAGGCTGTCTCCTCAGGTTGCCTCCTCAAAATGAAGTTGCCTGTTAGGC
M K L P V R

70 80 90 100 110 120
TGTTGGTGCTGATGTTCTGGATTCTGCTTCCAGCAGTGATGTTTTGATGACCCAAACTC
L L V L M F W I P A S S S D V L M T Q T
1

130 140 150 160 170 180
CACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGCAGATCTAGTCAGA
P L S L P V S L G D Q A S I S C R S S Q

190 200 210 220 230 240
CCATTGTACATAGTAATGGAAACACCTATTTACAATGGTACCTCCAGAAACCACGCCAGT
S I V H S N G N T Y L E W Y L Q K P G Q
CDR1

250 260 270 280 290 300
CTCCAAAGCTCCTGATCTCCAAAGTTTCCAACCCATTTTCTGCGGTCCCAGACAGGTTCA
S P K L L I S K V S N R F S G V P D R F
CDR2

310 320 330 340 350 360
GTGGCAGTGGATCAGGGACAGATTTACACTCAAGATCAGCAGACTCGAAGCTCAGCATC
S G S G S G T D F T L K I S R V E A E D

370 380 390 400 410 420
TGGGAGTTTATTACTGCTTTCAAGGTTTACATGTTCCATTACGTTCCGCTCGGGACAA
L G V Y Y C F Q G S H V P F T F G S G T
CDR3

430
AGTTGGAAATAAAA
K L E I K

*Kirby, Lades, Gale,
Baker & Potvin*

Figure 4:

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10 20 30 40 50 60
TTGACAGAGGAGGCCAGTCTGGATTCCGATTCCCAGTTCCTCACATTTCAGTGATCAGCACT

70 80 90 100 110 120
GAACACGGACCCTCACCATGAAGCTTGGGGCTCAGCTTGATTTTCCTTGTCCTTGTTTAA
M N L G L S L I F L V L V L

130 140 150 160 170 180
AAGGTGTCCAGTGTGAAGTGAAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAG
K G V Q C E V K L V E S G G G L V Q P G
1

190 200 210 220 230 240
GGTCCCTGAAACTCTCCTGTGCAACCTCTGGATTCACTTTCAGTGAATACATGTATT
G S L K L S C A T S G F T F S D Y Y M Y
CDR1

250 260 270 280 290 300
GGGTTGCCAGACTCCAGAGAAGAGCCTGGAGTGGOTCCGATACATTAGTAATGOTGCTG
W V R Q T P E K R L E W V A Y I S N G G

310 320 330 340 350 360
GTAGTAGCCATTATGTAGACAOTGTAAAGGGCCGATTACCATCTCCAGAGACAATGCCA
G S S H Y V D S V K G R F T I S R D N A
CDR2

370 380 390 400 410 420
AGAACACCCTGTACCTGCAAATGAGCCCTCTOAGGTCTGAGGACACAGCCATGTATCACT
K N T L Y L Q M S R L R S E D T A M Y H

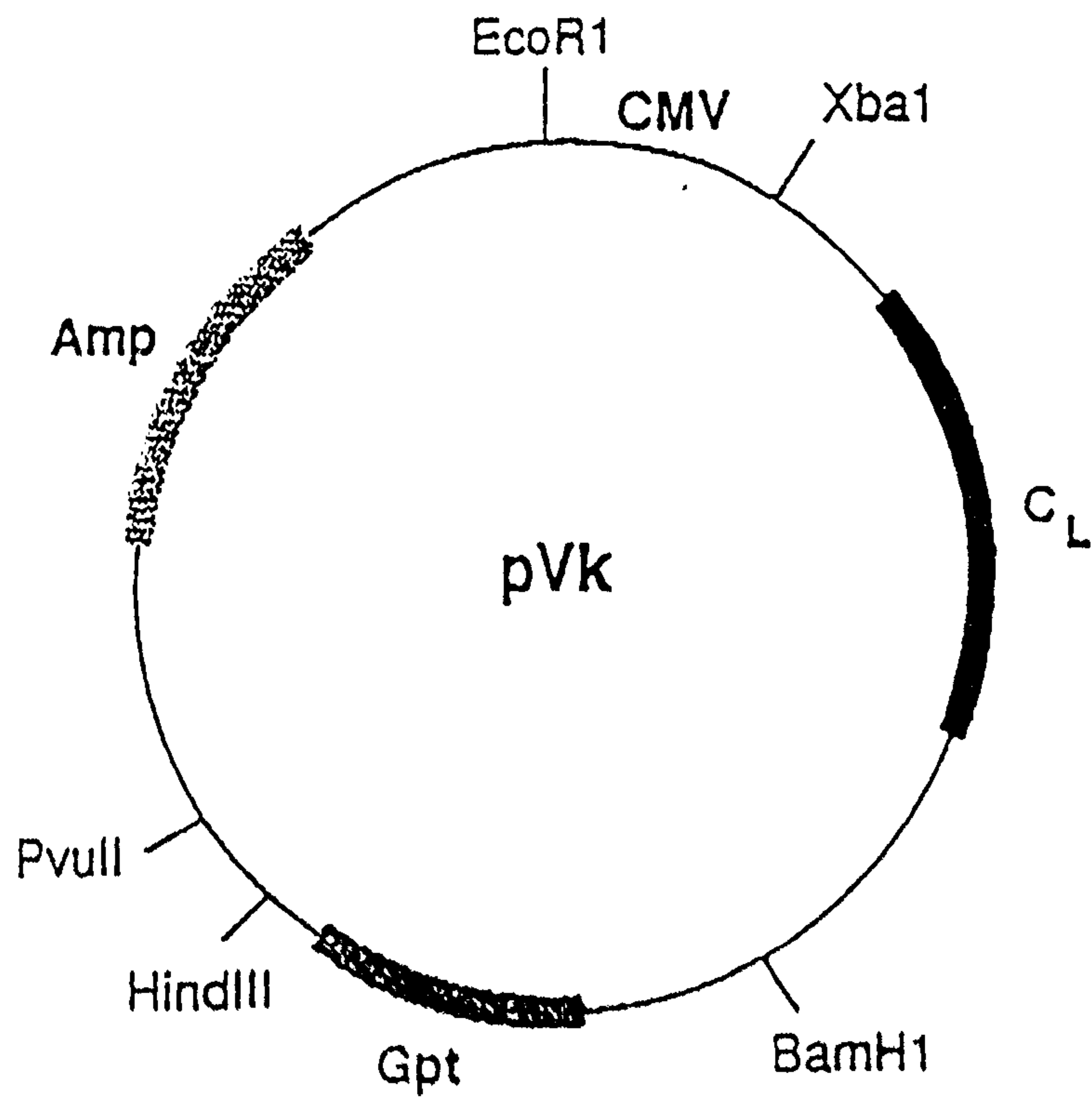
430 440 450 460 470 480
CGGCAAGGGGATGGATTACGGGGCCTCGTTTCCTTACTGGGGCCAGCGGACTCTGOTCA
C A R G M D Y G A W F A Y W G Q G T L V
CDR3

490
CTGTCTCTGCA
T V S A

*Kirby, Lades, Gale,
Baker & Potvin*

Figure 5:

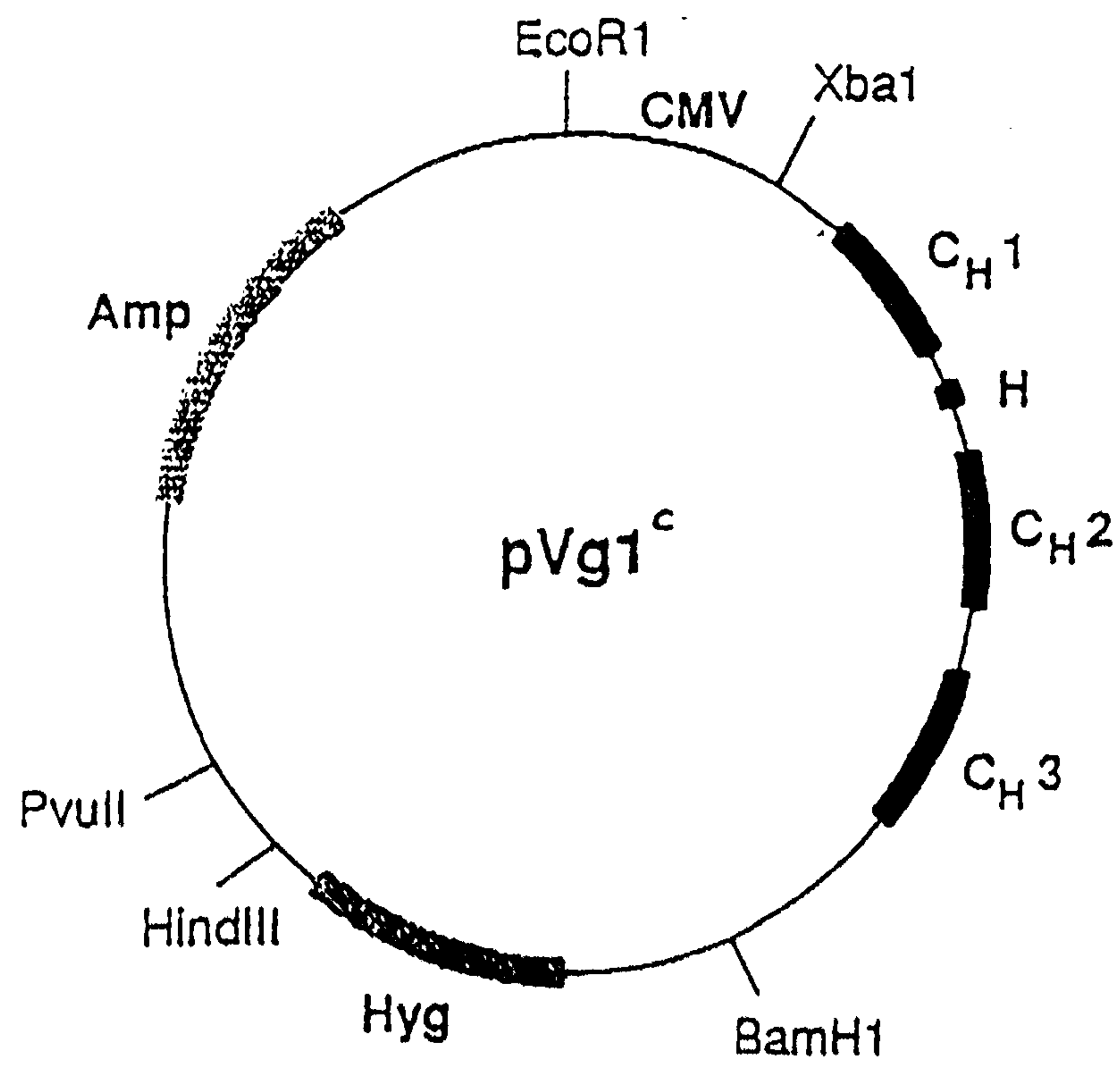
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Figure 6:

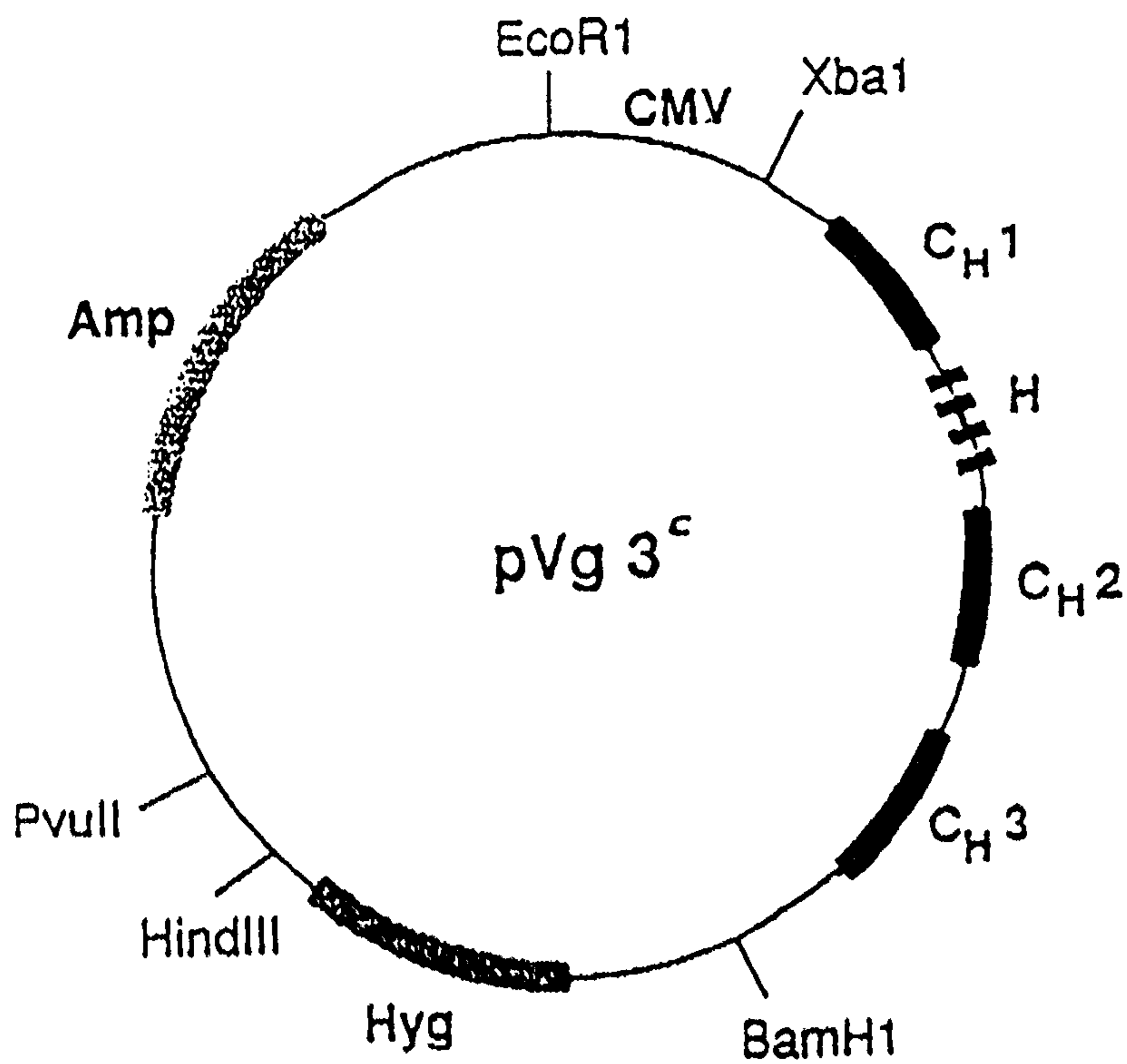
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Figure 7:

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Baker & Potvin*

Figure 8:

8/42

10 20 30 40 50 60
TCTAGACCACCATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCCTGCTT
M K L P V R L L V L M F W I P A

70 80 90 100 110 120
CCAGCAGTGATGTTTTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATC
S S S D V L M T Q T P L S L P V S L G D
1

130 140 150 160 170 180
AAGCCTCCATCTCTTGCAGATCTAGTCAGAGCATTGTACATAGTAATGGAAACACCTATT
Q A S I S C R S S Q S I V H S N G N T Y

190 200 210 220 230 240
TAGAATGGTACCTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTCCAAAGTTTCCA
L E W Y L Q K P G Q S P K L L I S K V S

250 260 270 280 290 300
ACCGATTTTCTCGGGTCCCAGACAGGTTTCAGTCGCACTCGATCAGGGACAGATTTACAC
N R F S G V P D R F S G S G S G T D F T

310 320 330 340 350 360
TCAAGATCAGCAGAGTCGAGGCTCAGGATCTCGGAGTTTATTACTGCTTTCAAGGTTTAC
L K I S R V E A E D L G V Y Y C F Q G S

370 380 390 400 410 420
ATGTTCCATTACGTTCCGGCTCGGGGACAAACTTGGAAATAAAACGTAAGTAGACTTTTC
H V P F T F G S G T K L E I K

CTCTAGA

Kirby, Lades, Gale,
Baker & Potvin

Figure 9:

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10 20 30 40 50 60
TCTAGACCACCATGAACTTGGGGCTCAGCTTGATTTTCCTTGTCTTGTTTTAAAAGGTG
M N L G L S L I F L V L V L K G

70 80 90 100 110 120
TCCAGTGTGAACTGAAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCC
V Q C E V K L V E S G G G L V Q P G G S
1

130 140 150 160 170 180
TGAAACTCTCCTGTGCAACCTCTGGATTCACTTTCAGTGA CTATTACATGTATTGGCTTC
L K L S C A T S G F T F S D Y Y M Y W V

190 200 210 220 230 240
GCCAGACTCCAGAGAAGAGCCTGGACTGGGTCCGATACATTAGTAATGCTGCTGCTAGTA
R Q T P E K R L E W V A Y I S N G G G S

250 260 270 280 290 300
GCCATTATGTAGACAGTGTAAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACA
S H Y V D S V K G R F T I S R D N A K N

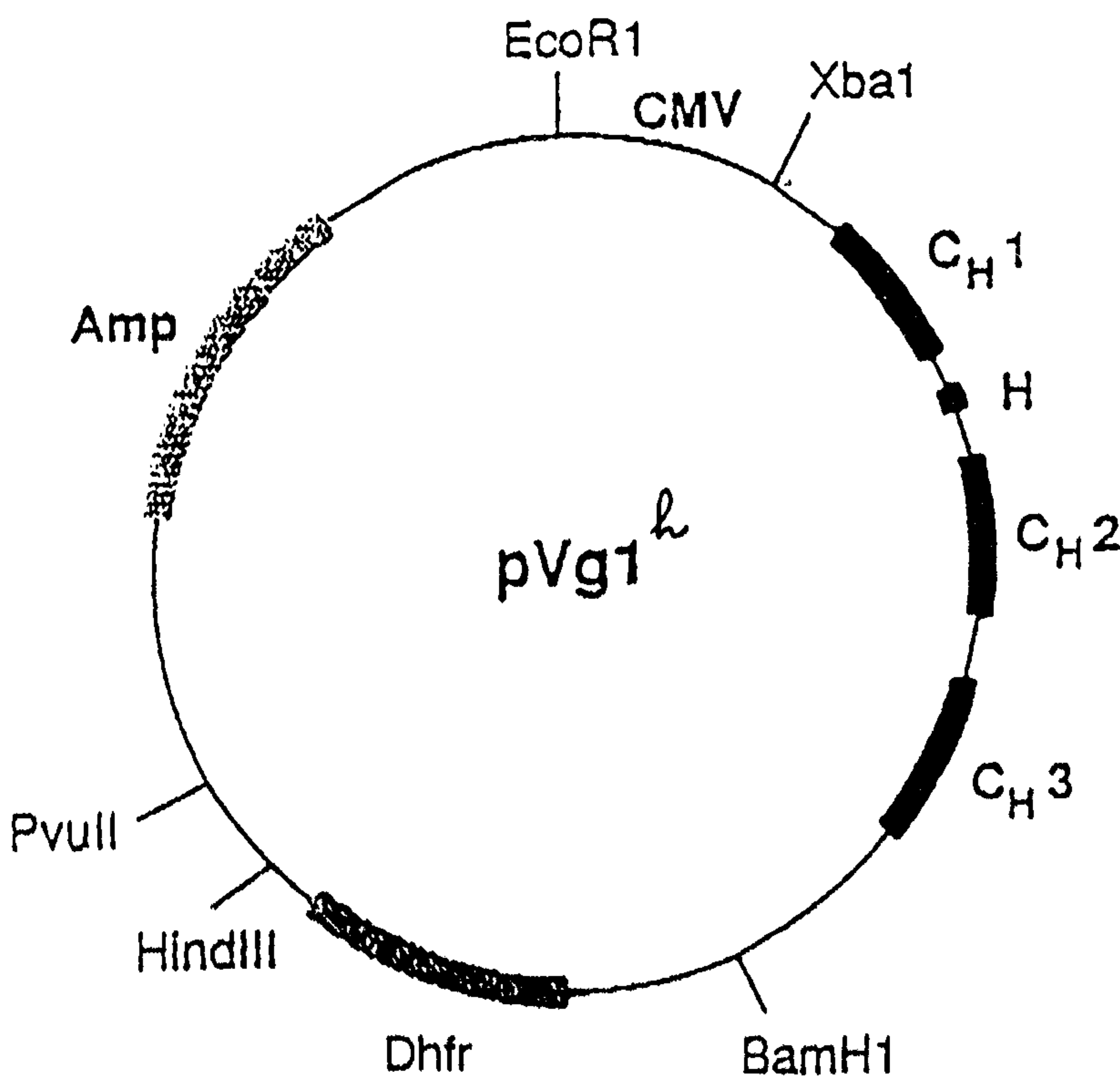
310 320 330 340 350 360
CCCTGTACCTGCAAATGAGCCGTCTGAGGTCTGAGGACACAGCCATGTATCACTCCGCAA
T L Y L Q M S R L R S E D T A M Y H C A

370 380 390 400 410 420
GGGGCATGGATTACGGGGCCTGCTTTGCTTACTCGGGCCAGGGGACTCTGCTCACTGTCT
R G M D Y G A W F A Y W G Q Q T L V T V

↓ 430 440
CTGCAGGTGAGTCCTAACTTCTAGA
S A

Figure 10:

10/42



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Figure 11:

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10 20 30 40 50
H-hu-BR55-2/1: EVQLLESGGG LVQPGGSLRL SCAASGFTFS DYYMYWVRQA PGKGLEWVAY
or H-hu-BR55-2/2: E R
or H-hu-BR55-2/3: E R
60 70 80 90 100
ISNCGGSSHY VDSVKGRFTI SRDNSKNTLY LQMNSLRAED TALYYCARGM
A H
H
110 119
DYCAWFAYWG QGTLVTVSS

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Baker & Potvin

Figure 12:

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1	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S
1	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S
21	I	S	C	R	S	S	Q	S	I	V	H	S	N	G	N	T	Y	L	E	W
21	I	S	C	R	S	S	Q	S	L	L	H	S	D	G	F	D	Y	L	N	W
41	Y	L	Q	K	P	G	Q	S	P	Q	L	L	I	S	K	V	S	N	R	F
41	Y	L	Q	K	P	G	Q	S	P	Q	L	L	I	Y	A	L	S	N	R	A
61	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I
61	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I
81	S	R	V	E	A	E	D	V	G	V	Y	Y	C	F	Q	G	S	H	V	P
81	S	R	V	E	A	E	D	V	G	V	Y	Y	C	M	Q	A	L	Q	A	P
101	F	T	F	G	Q	G	T	K	L	E	I	K								
101	I	T	F	G	Q	G	T	R	L	E	I	K								

Figure 13:

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1	E V Q L L E S G G G L V Q P G G S L R L
1	E V Q L L E S G G G L V Q P G G S L R L
21	S C A A S G F T F S <u>D Y Y M Y</u> W V R Q A
21	S C A A S G F T F S S S A M S W V R Q A
41	P <u>E</u> K <u>R</u> L E W V A Y <u>I S N G G G S S H Y</u>
41	P G K G L E W V A W K Y E N G N D K H Y
61	<u>V D S V K G</u> R F T I S R <u>D</u> <u>N</u> <u>A</u> K N T L Y
61	A D S V N G R F T I S R N D S K N T L Y
81	L <u>Q</u> M N S L <u>R</u> A E D T A L Y <u>H</u> C A R <u>G</u>
81	L L M N S L Q A E D T A L Y Y C A R D A
100	<u>M D Y</u> <u>G A W F A Y</u> <u>W</u> G Q G T L V T V
101	G P Y V S P T F F A H Y G Q G T L V T V
118	S S
121	S S

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Figure 14:

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jb37

10	20	30	40	50	60
TATATCTAGA	CCACCATGAA	GTTGCCTGTT	AGGCTGTTGG	TGCTGATGTT	CTGGATTTCCT
70	80	90	100		
GCTTCCAGCA	GTGATATTGT	GATGACCCAA	TCTCCACTCT	CCCTGCCT	

jb 38

10	20	30	40	50	60
TATAGGTACC	ATTCTAAATA	CGTGTTTCCA	TTACTATGTA	CAATGCTCTG	ACTAGACCTG
70	80	90	100		
CAAGAGATGG	AGGCTGCCTC	TCCAGGAGTG	ACAGGCAGGG	AGAGTGGA	

jb39

10	20	30	40	50	60
TATAGGTACC	TTCAGAAACC	AGGCCAGTCT	CCACAGCTCC	TGATCTCCAA	AGTTTCCAAC
70	80	90	100	110	120
CGATTTTCTG	GGGTCCCAGA	CAGGTTCACT	GGCACTGCAT	CAGGCACAGA	TTTCACACTC
130					
AACATCAGCA	GAG				

jb40

10	20	30	40	50	60
TATATCTAGA	GCAAAAGTCT	ACTTACGTTT	TATTTCCAAC	TTTGTCCCCT	GGCCGAACGT
70	80	90	100	110	120
GAATGGAACA	TGTGAACCTT	GAAAGCAGTA	ATAAACTCCC	ACATCCTCAG	CCTCCACTCT
130					
GCTGATCTTG	AG				

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Figure 15:

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mc108

10	20	30	40	50	60
TATATCTAGA	CCACCATGAA	CTTCGGGCTA	AGCTTGATTT	TCCTTGTCCT	TGTTTTAAAA
70	80	90	100	110	120
GGTGTCCAGT	GTGAAGTGCA	ACTGCTGGAG	TCTGGGGGAG	GCTTAGTGCA	GCCTGCAGGA
130					
AGTCTACGAC	TC				

mc109

10	20	30	40	50	60
TATAGAGCTC	CCACCACCGT	TGCTAATGTA	TGCGACCCAC	TCCAGCCTCT	TTTCTGGAGC
70	80	90	100	110	120
CTGGCGAACC	CAGTACATGT	AATAATCACT	GAAAGTGAAT	CCAGAGGCTG	CACAGGAGAG
130					
TCGTAGACTT	CCT				

mc110

10	20	30	40	50	60
TATAGAGCTC	ACATTACGTA	GATTCCGTCA	AGGCCCCGATT	CACCATCTCC	AGAGATAATC
70	80	90	100	110	
CCAAGAACAC	CCTGTACCTG	CAOATGAACT	CACTGCGACC	TGAGGACACG	GCCTTATA

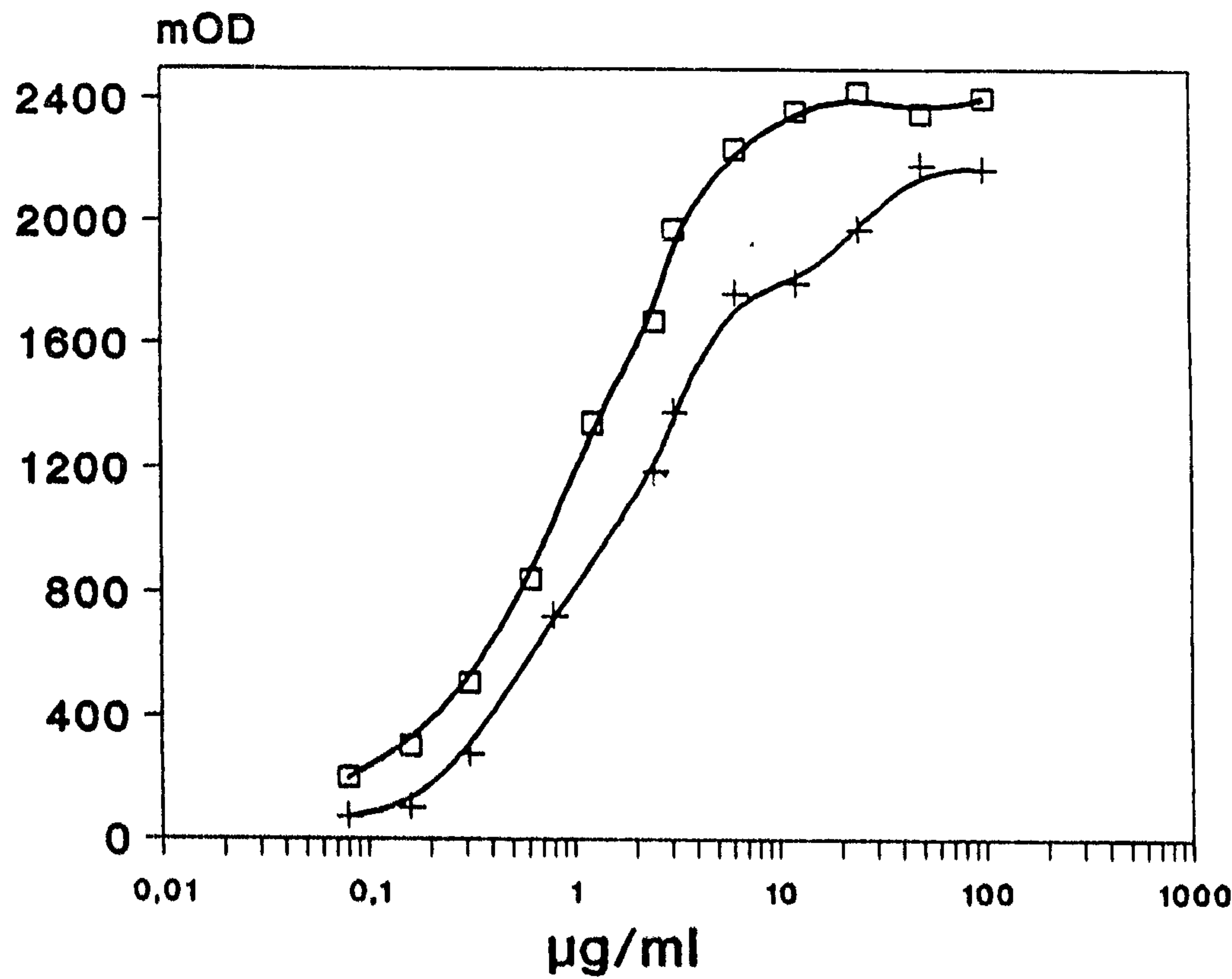
mc111

10	20	30	40	50	60
TATATCTAGA	AAAAAGCCAG	CTTACCTGAG	GACACGGTGA	CCAGGGTCCC	TTGGCCCCAG
70	80	90	100	110	
TATGCGAACC	ATGCCCCGTA	GTCCATCCCT	CTTGACACAGT	GATATAAGGC	CGTGTCTT

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Figure 16:

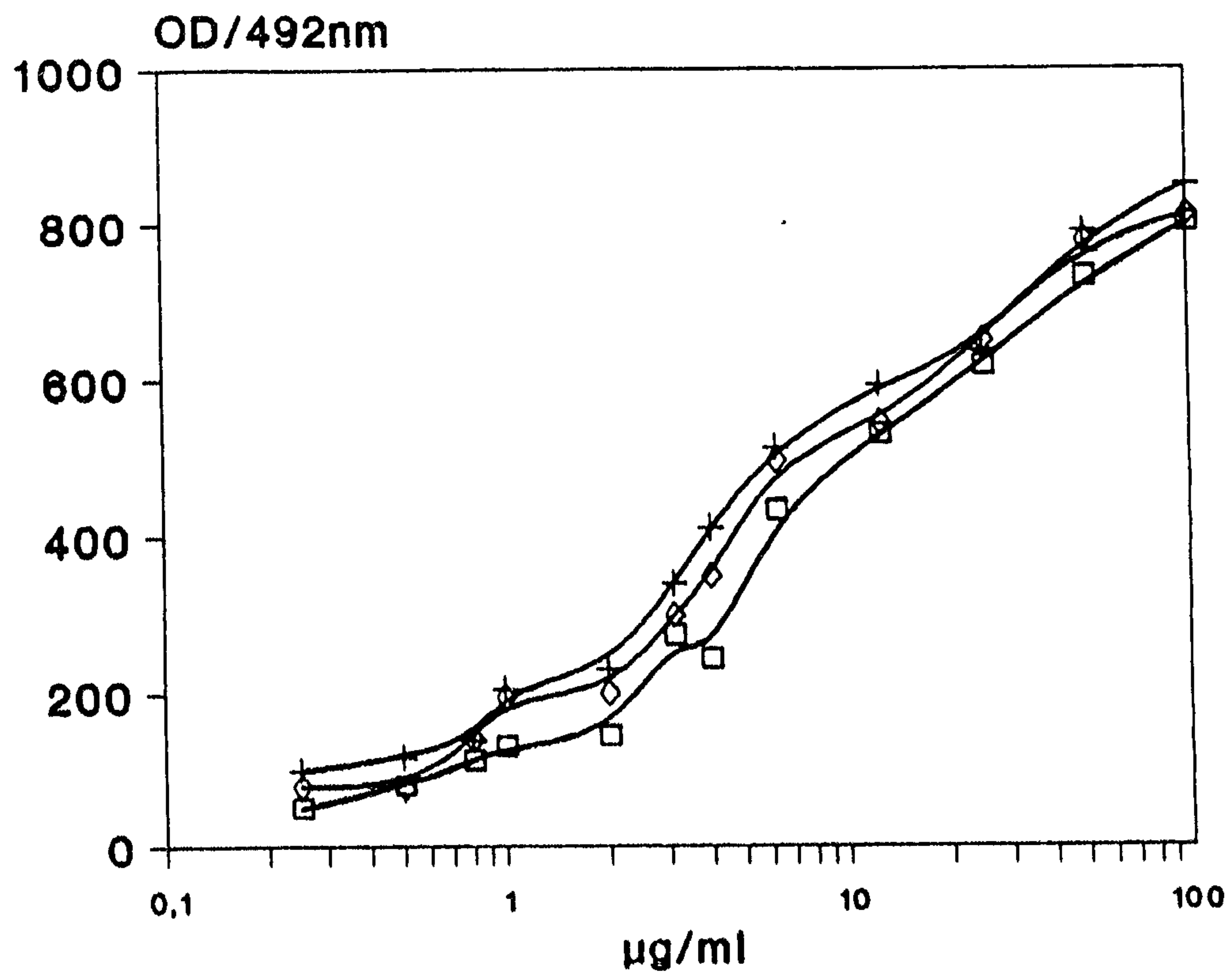
16/42



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Figure 17:

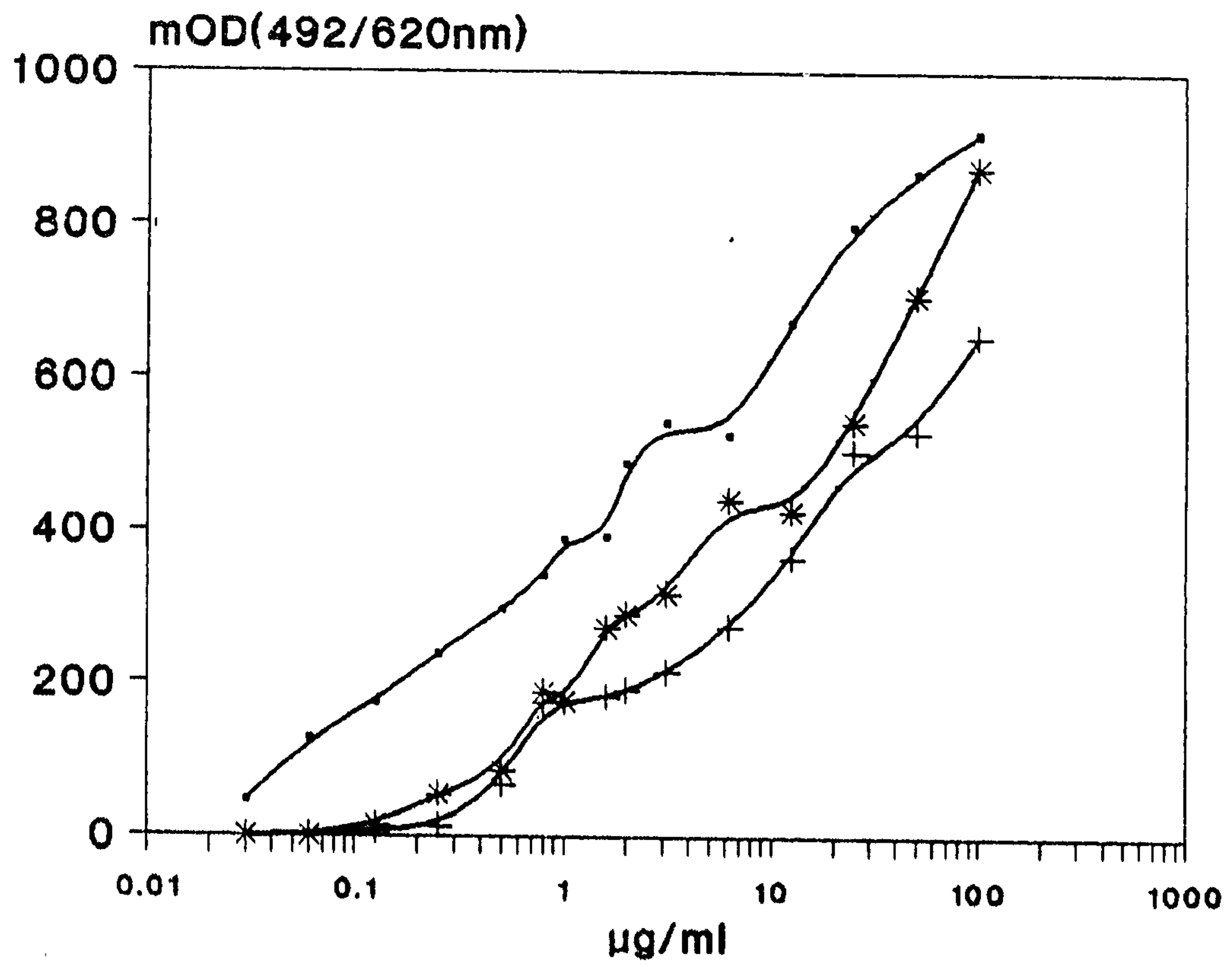
17/42



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Figure 18:

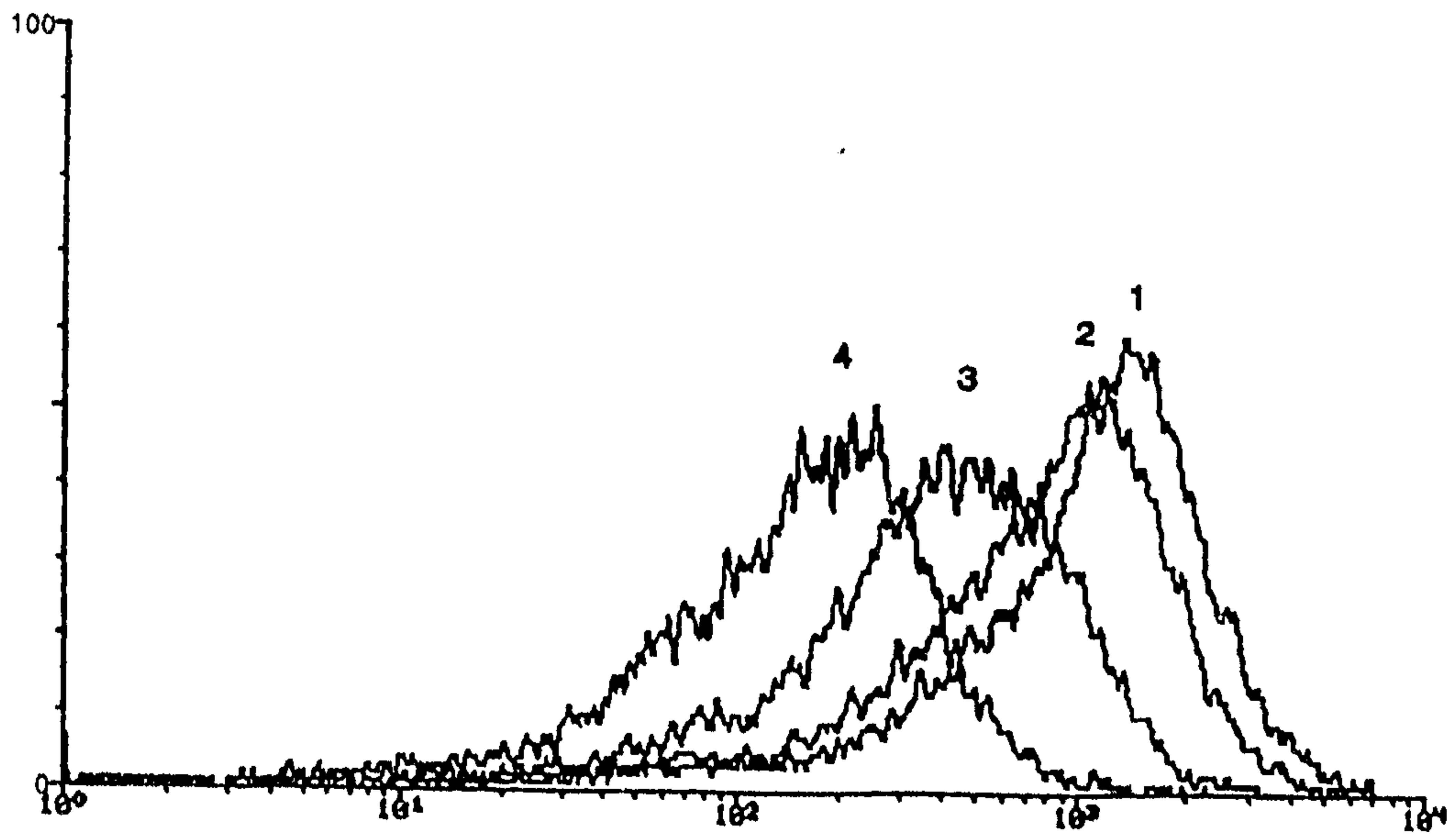
18/42



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Figure 19:

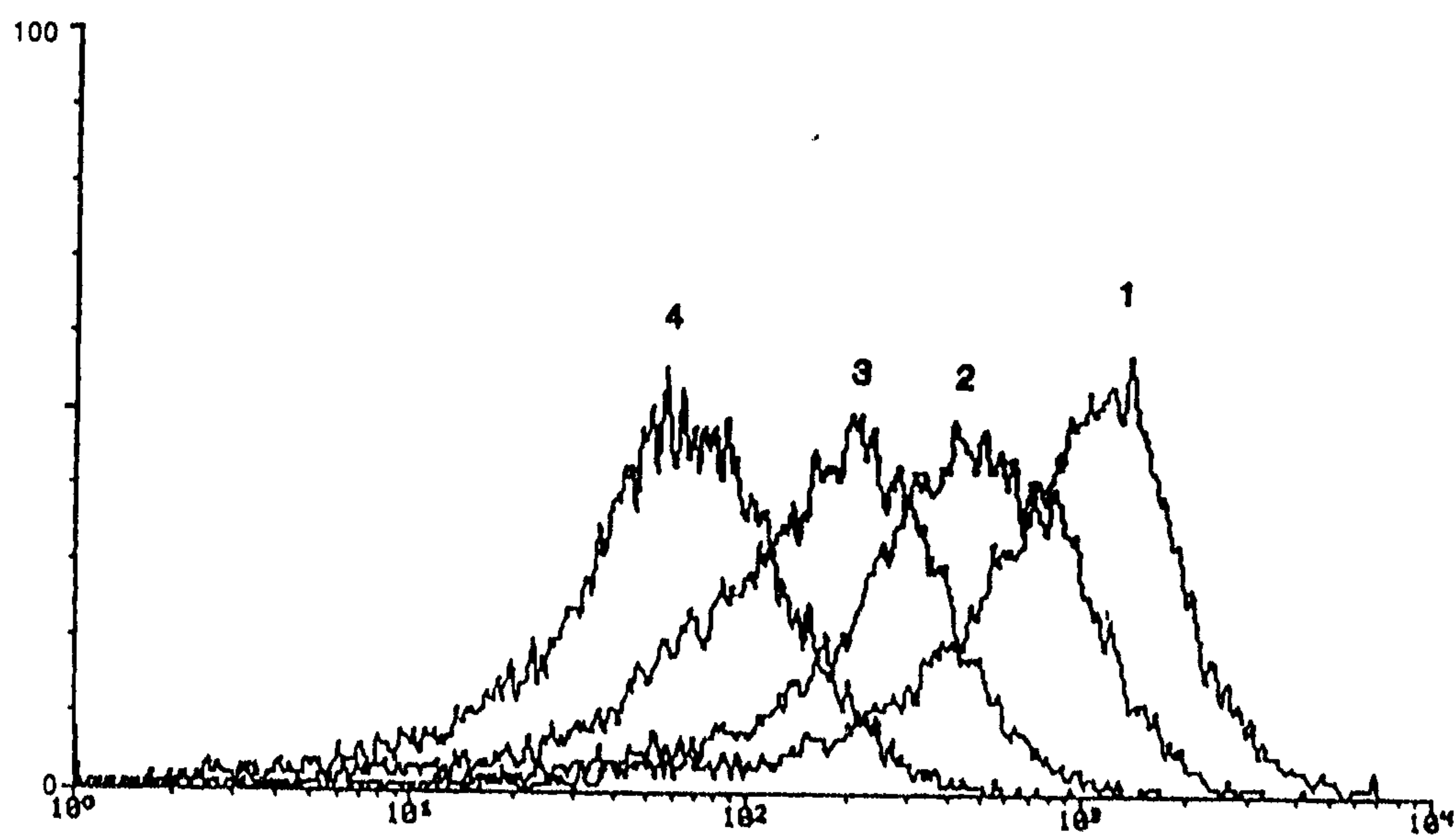
19/42



Kirby, Lades, Gale,
Baker & Potvin

Figure 20:

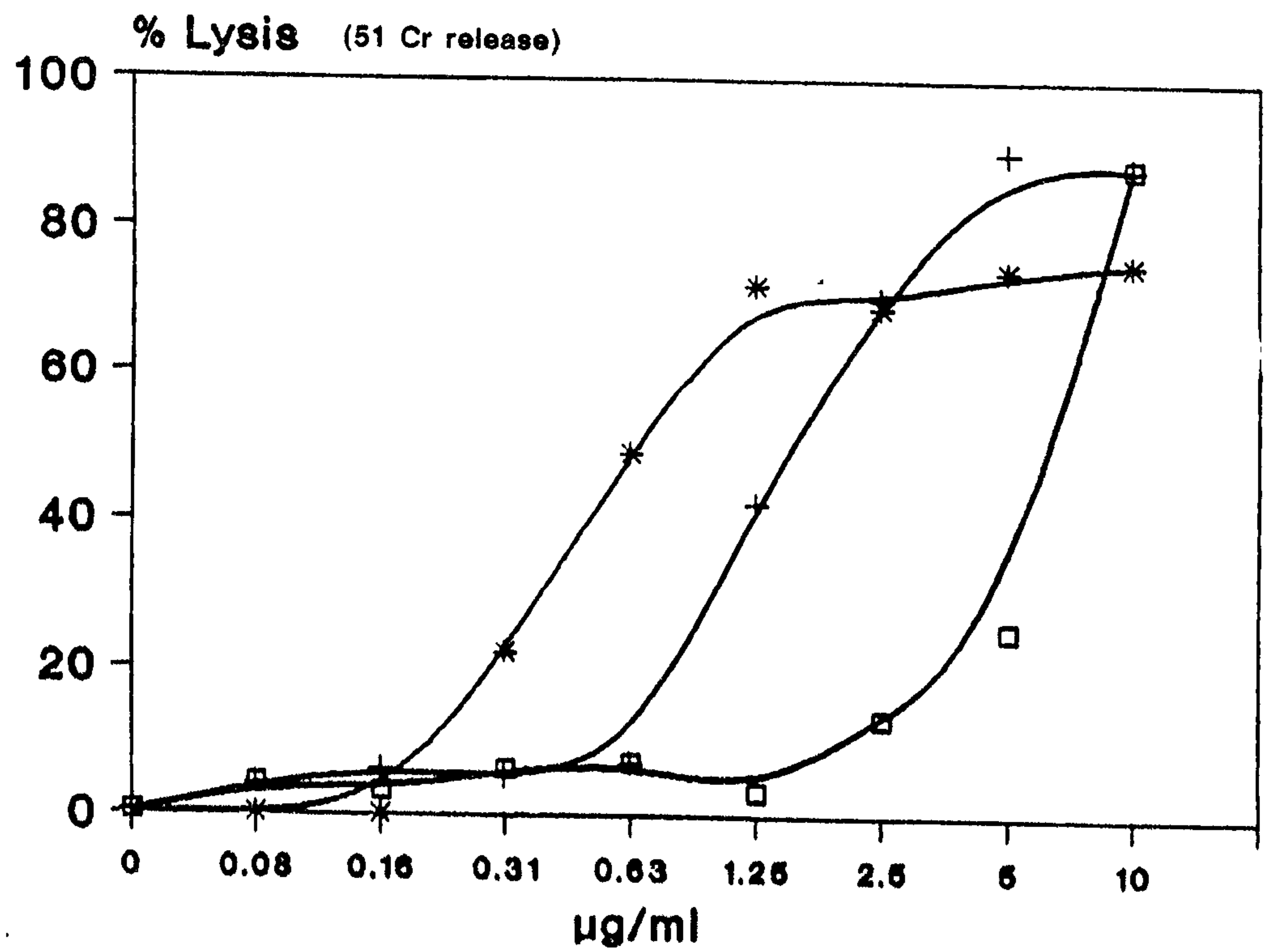
20/42



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Figure 21:

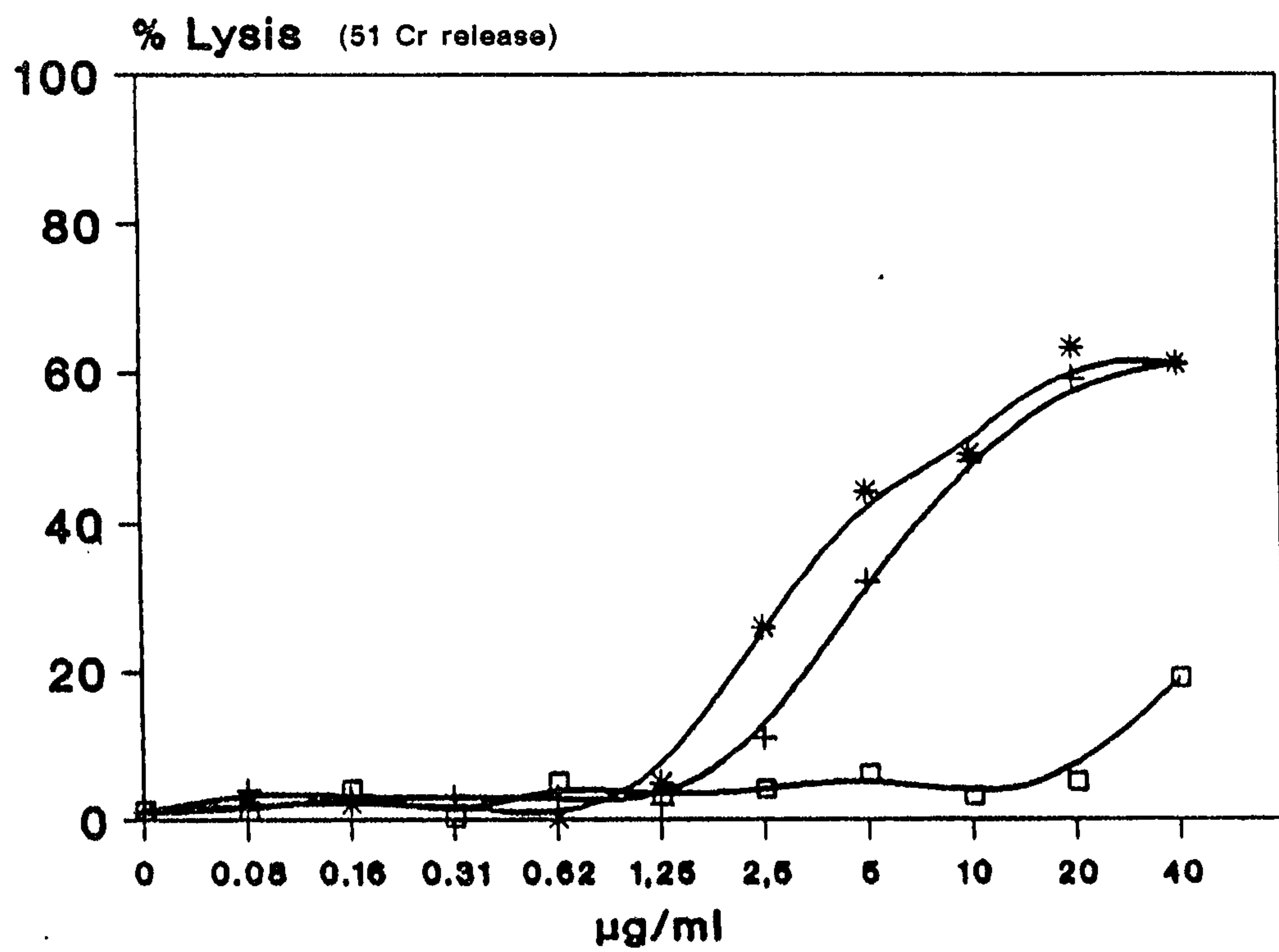
21/42



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Figure 22:

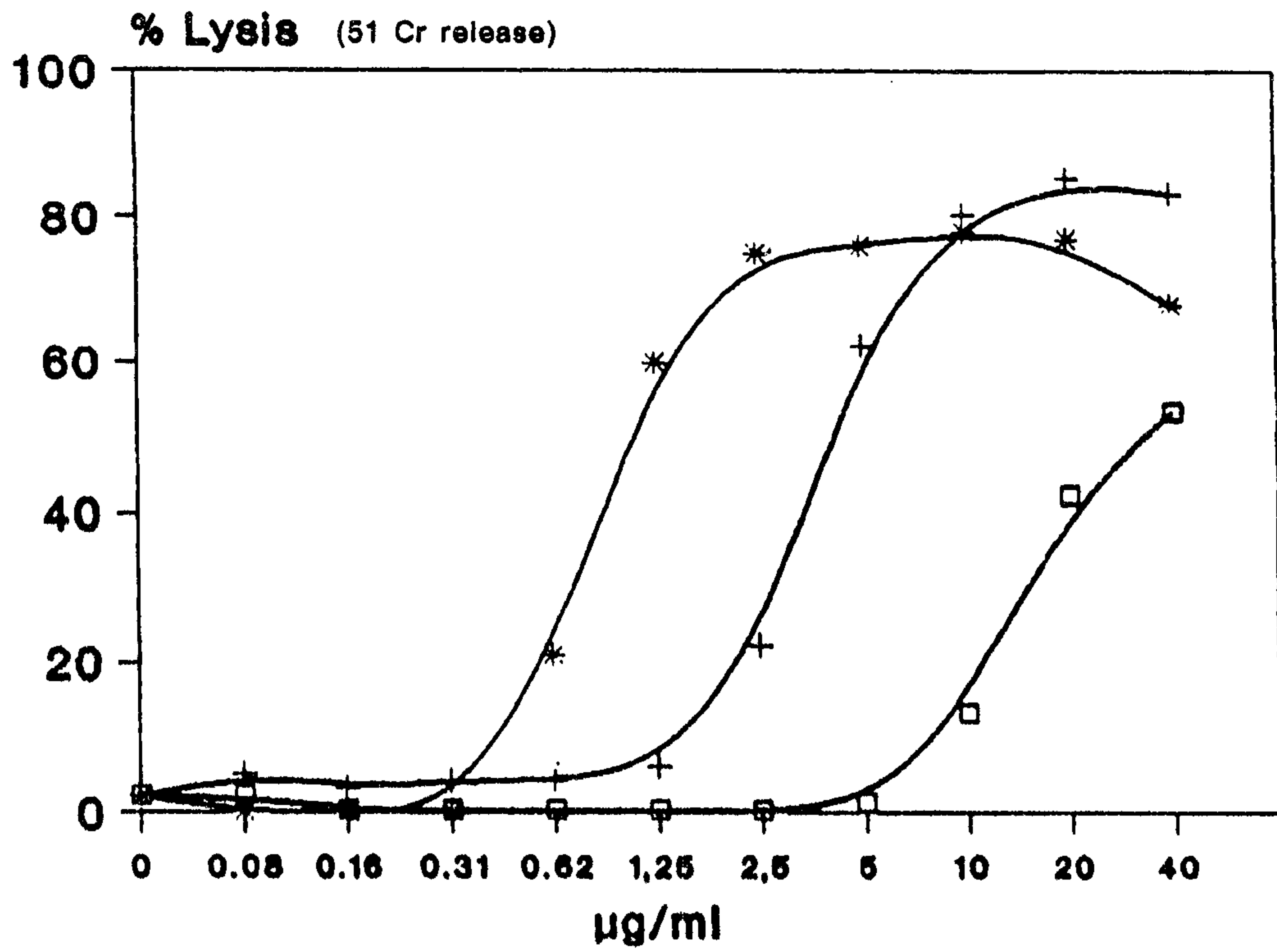
22/42



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Figure 23:

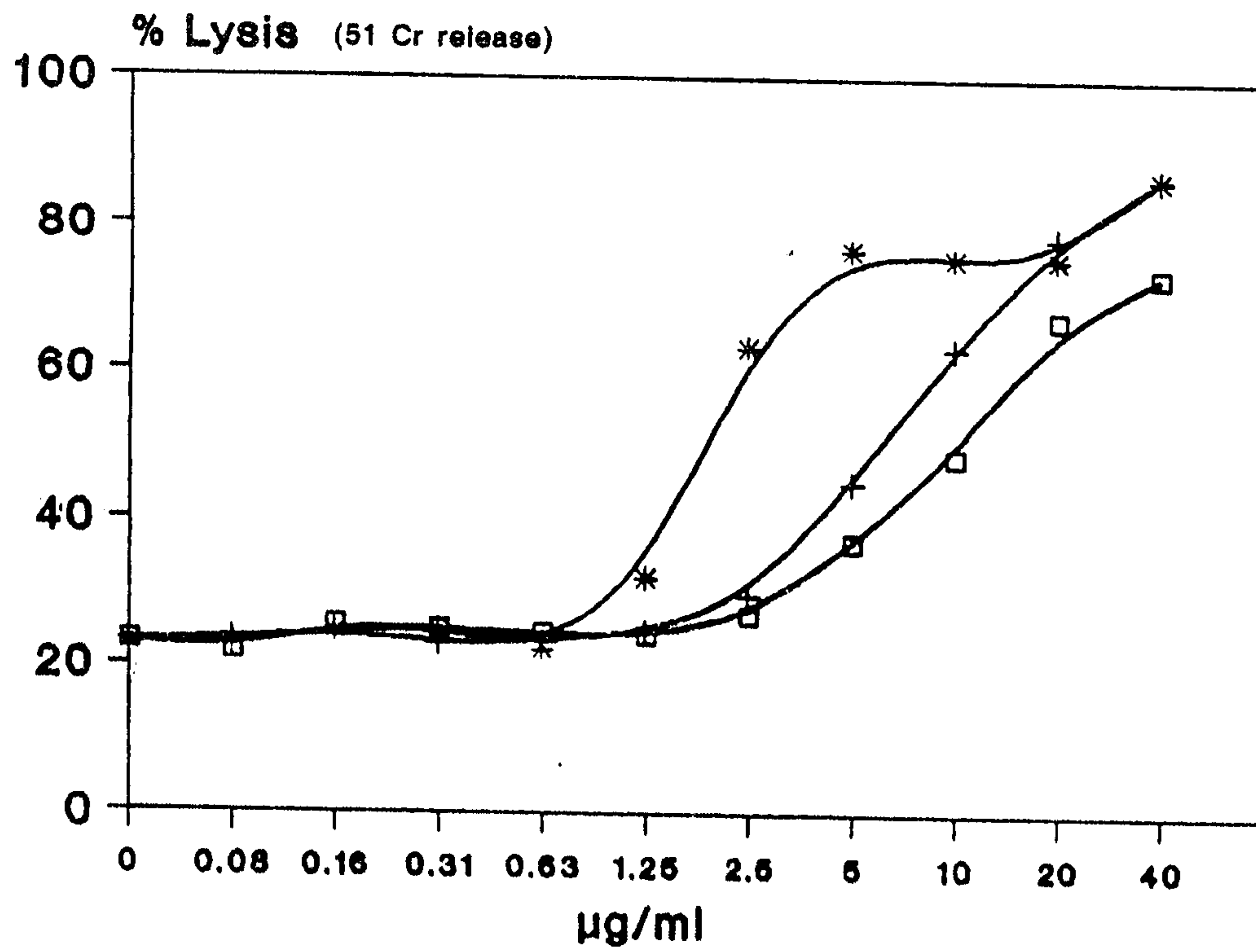
23/42



*Kirby, Lades, Gale,
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Figure 24:

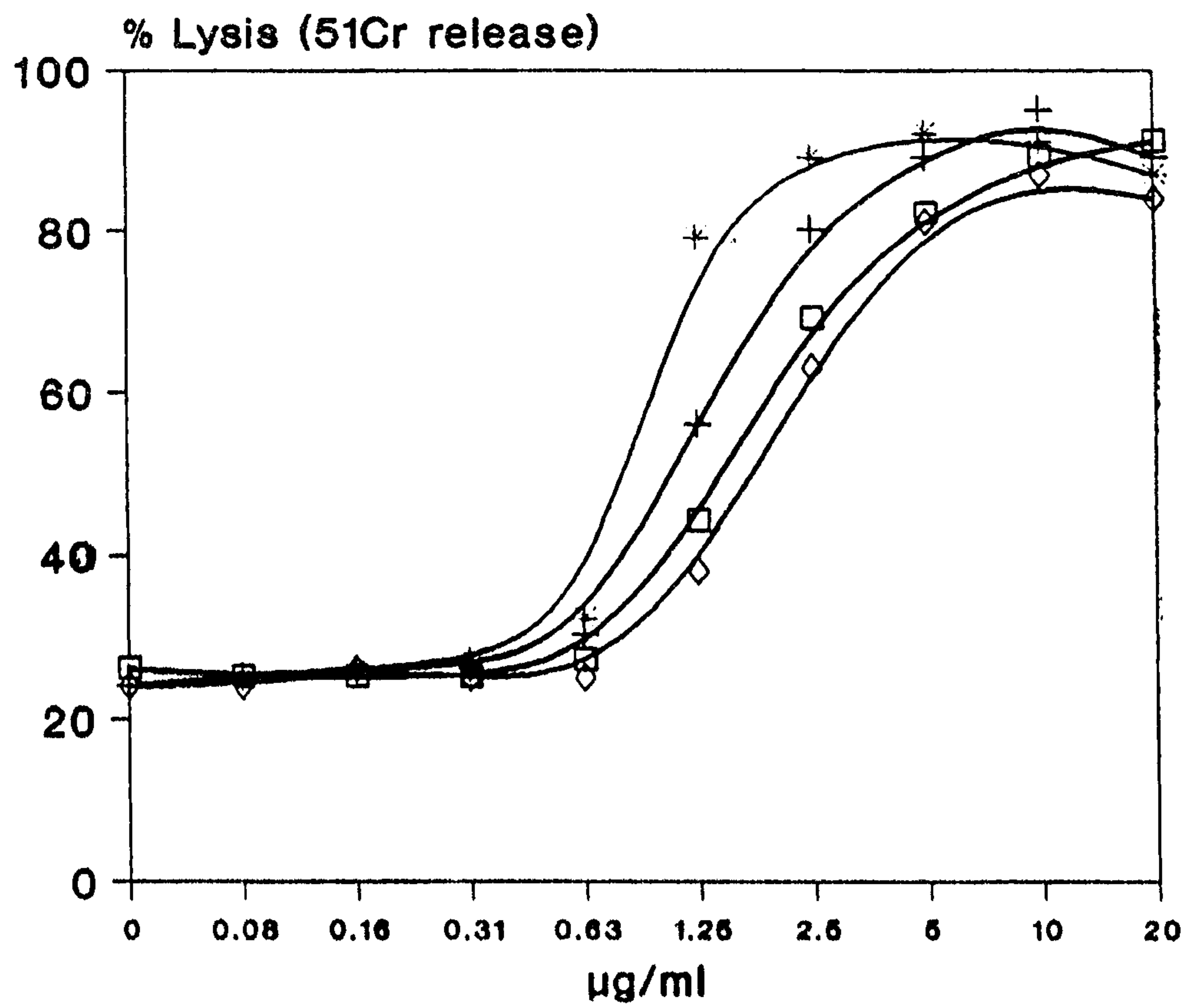
24/42



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Figure 25:

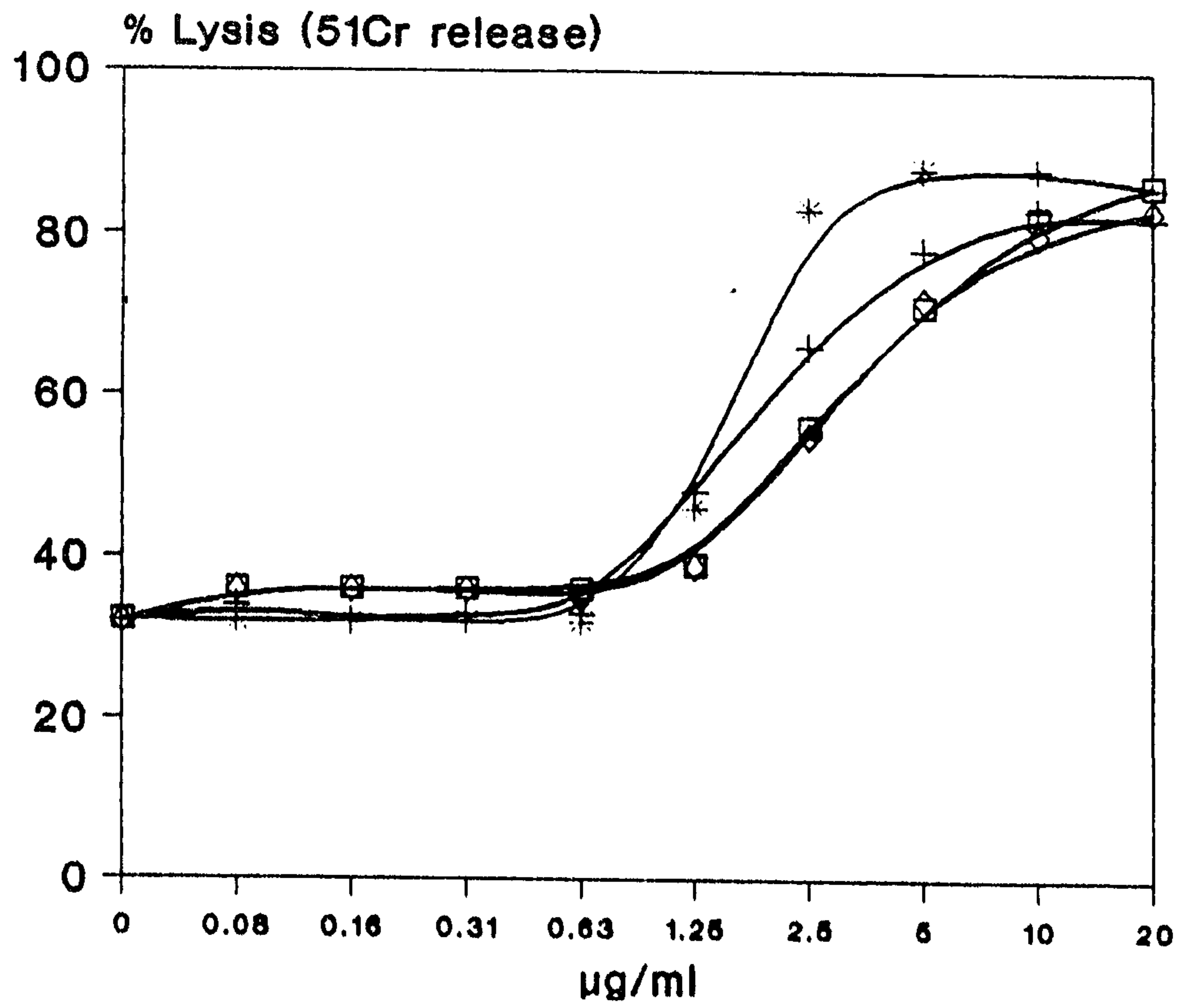
25/42



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Figure 26:

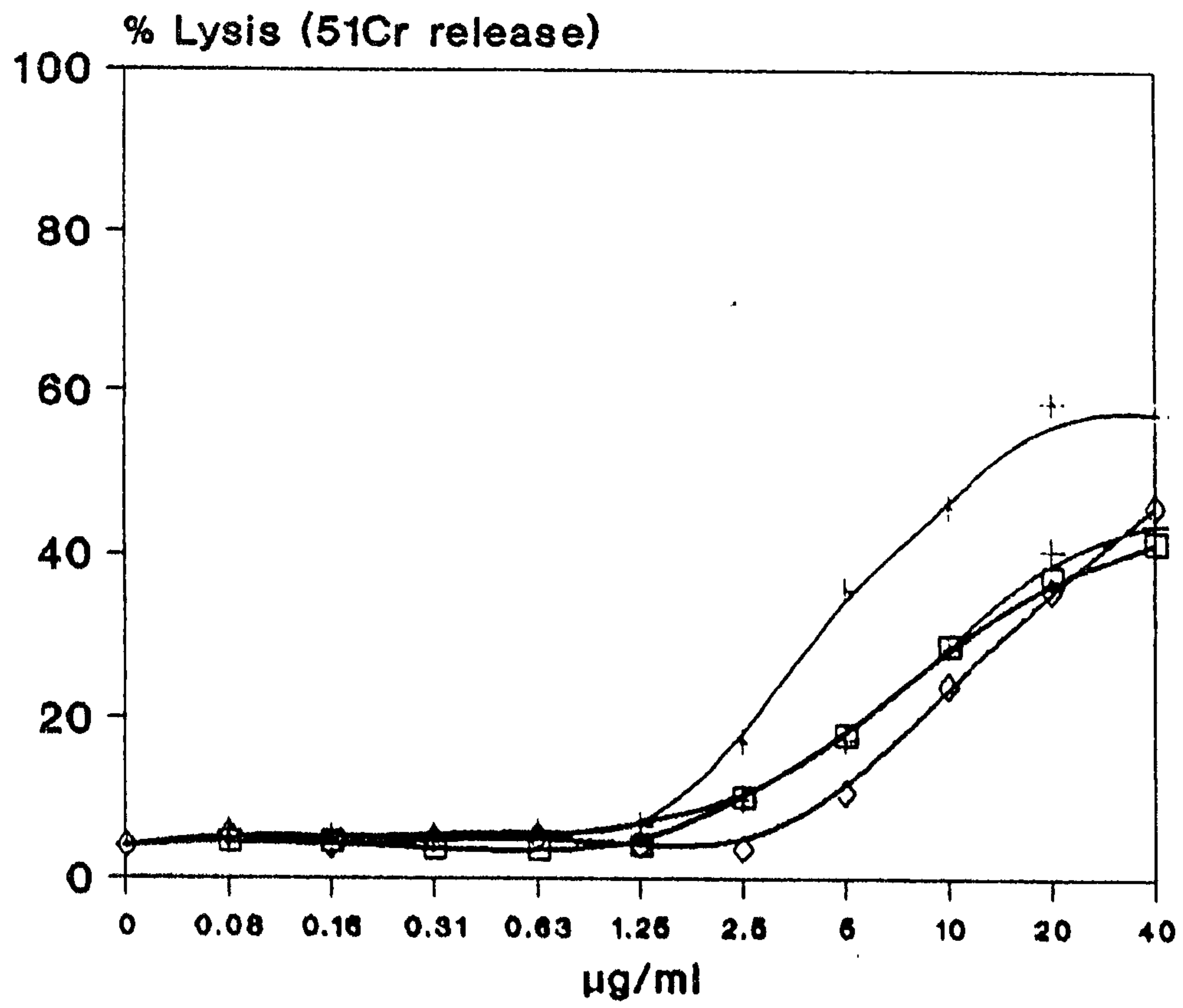
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Figure 27:

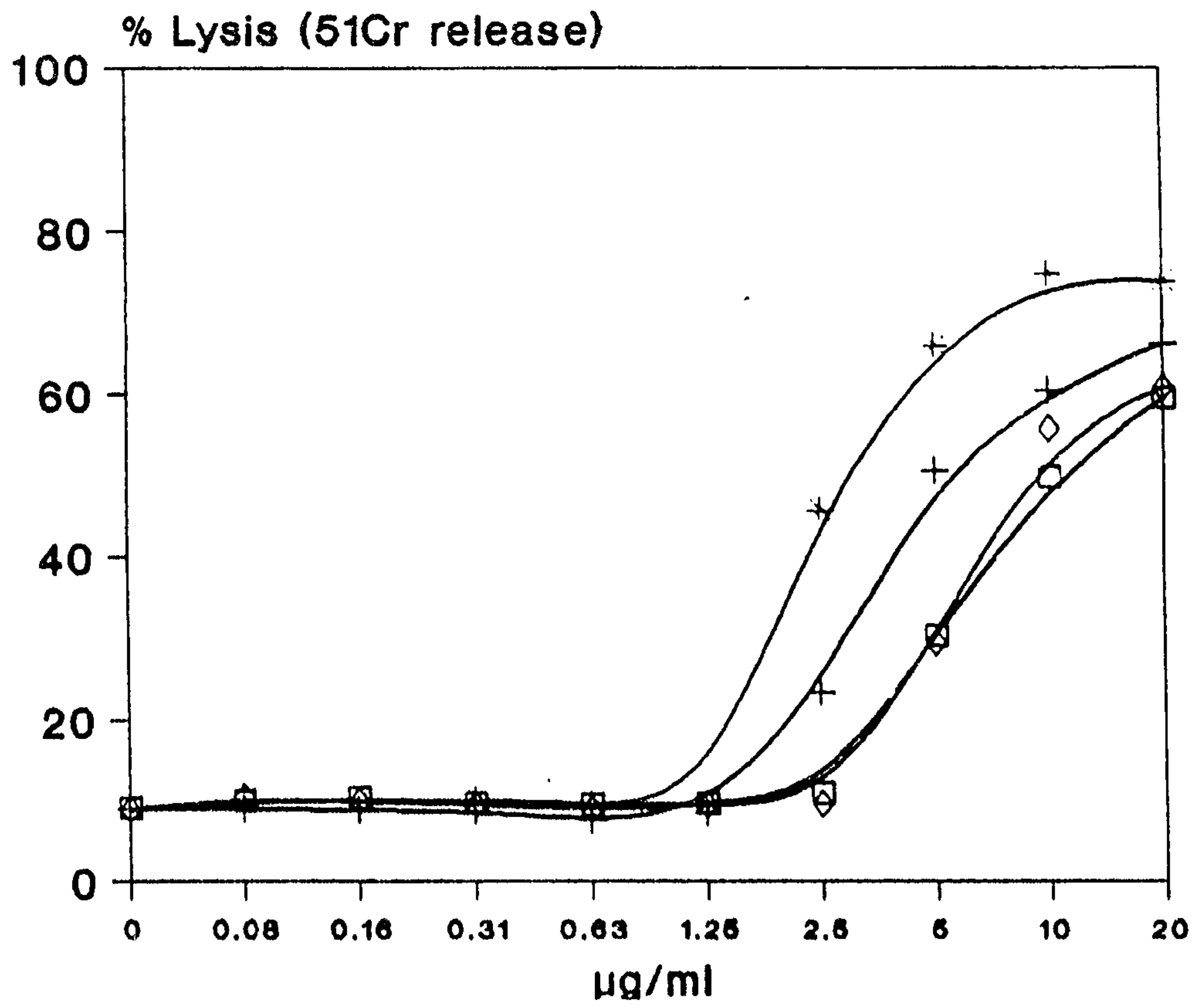
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Figure 28:

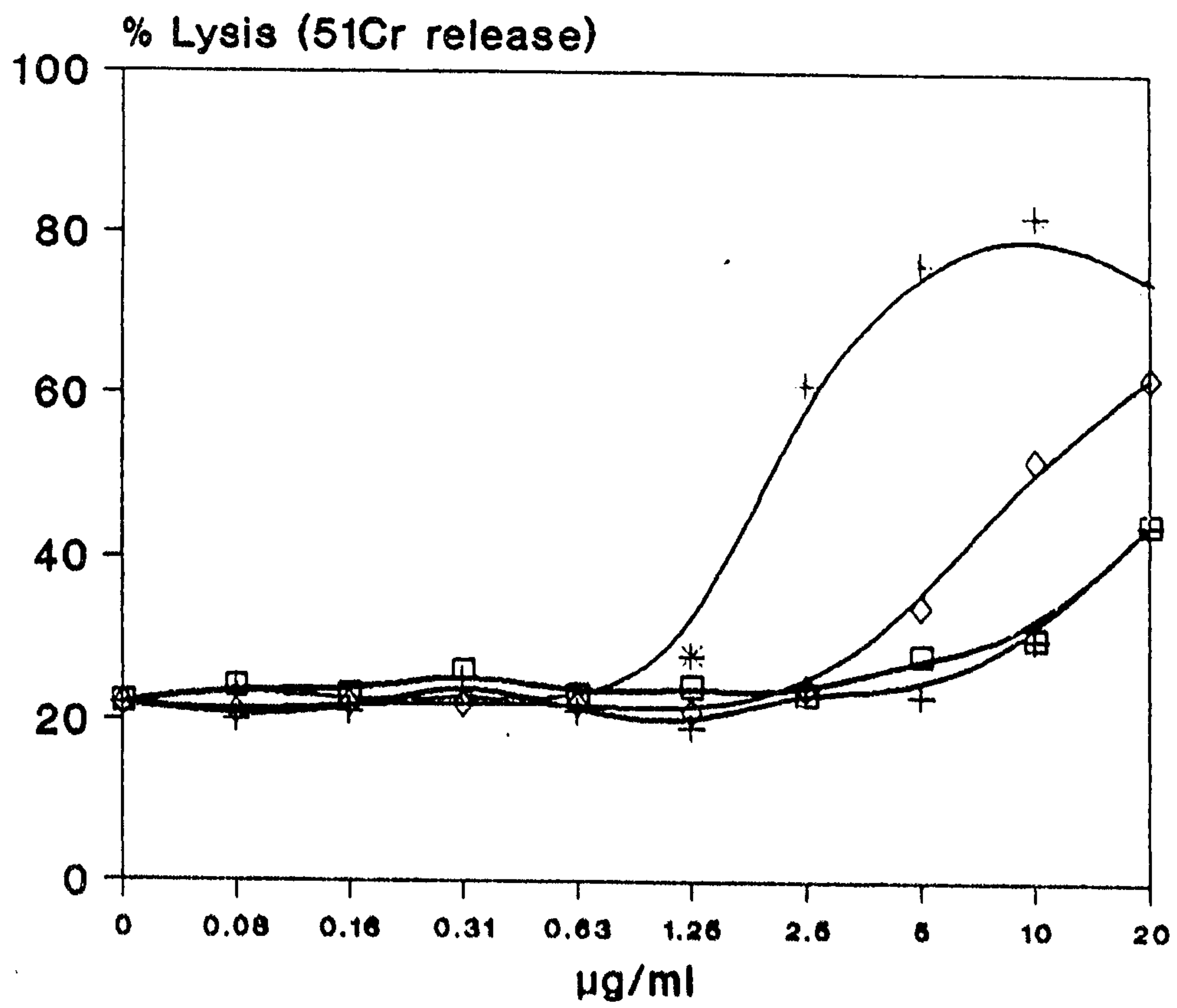
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Figure 29:

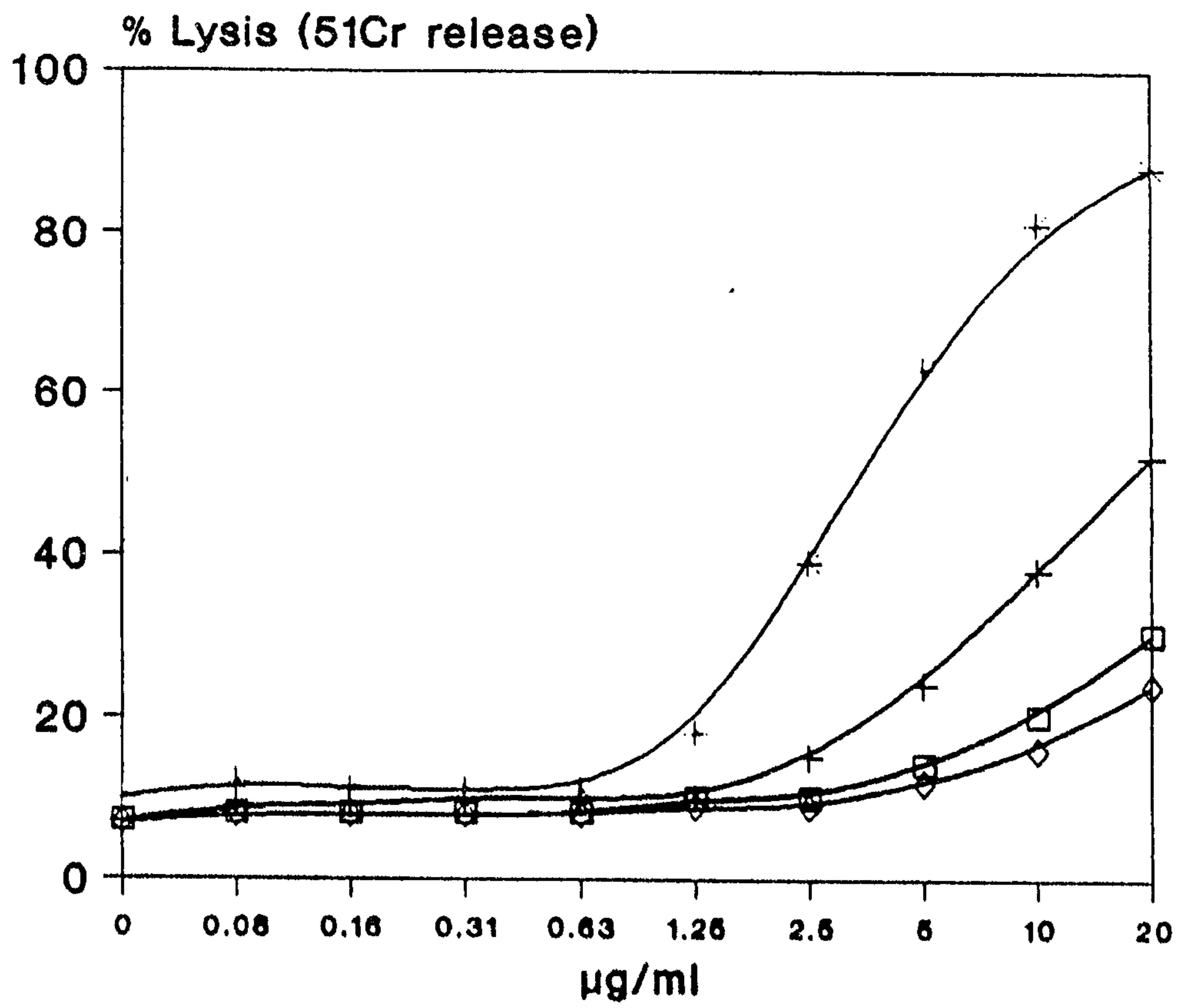
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Figure 30:

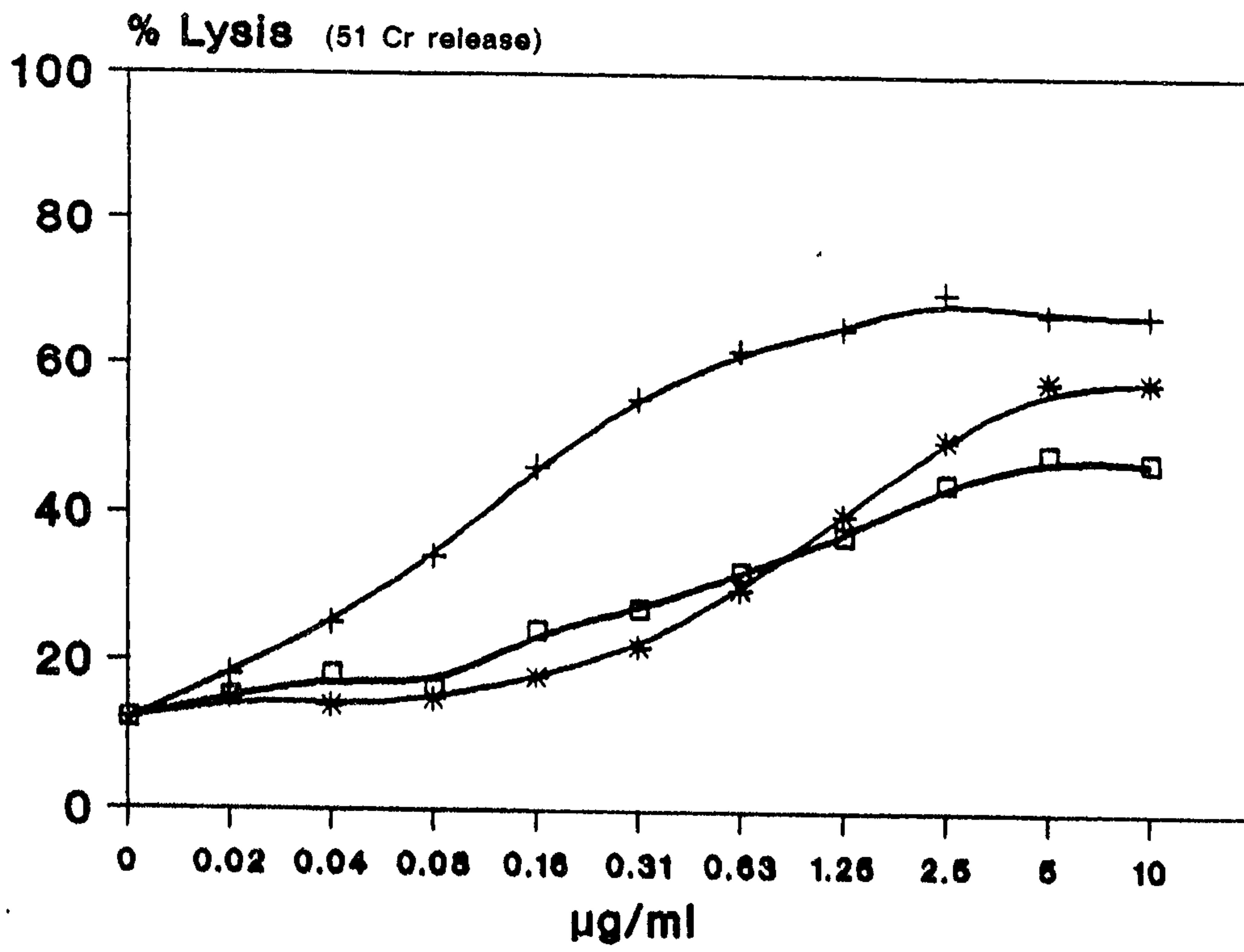
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Figure 31:

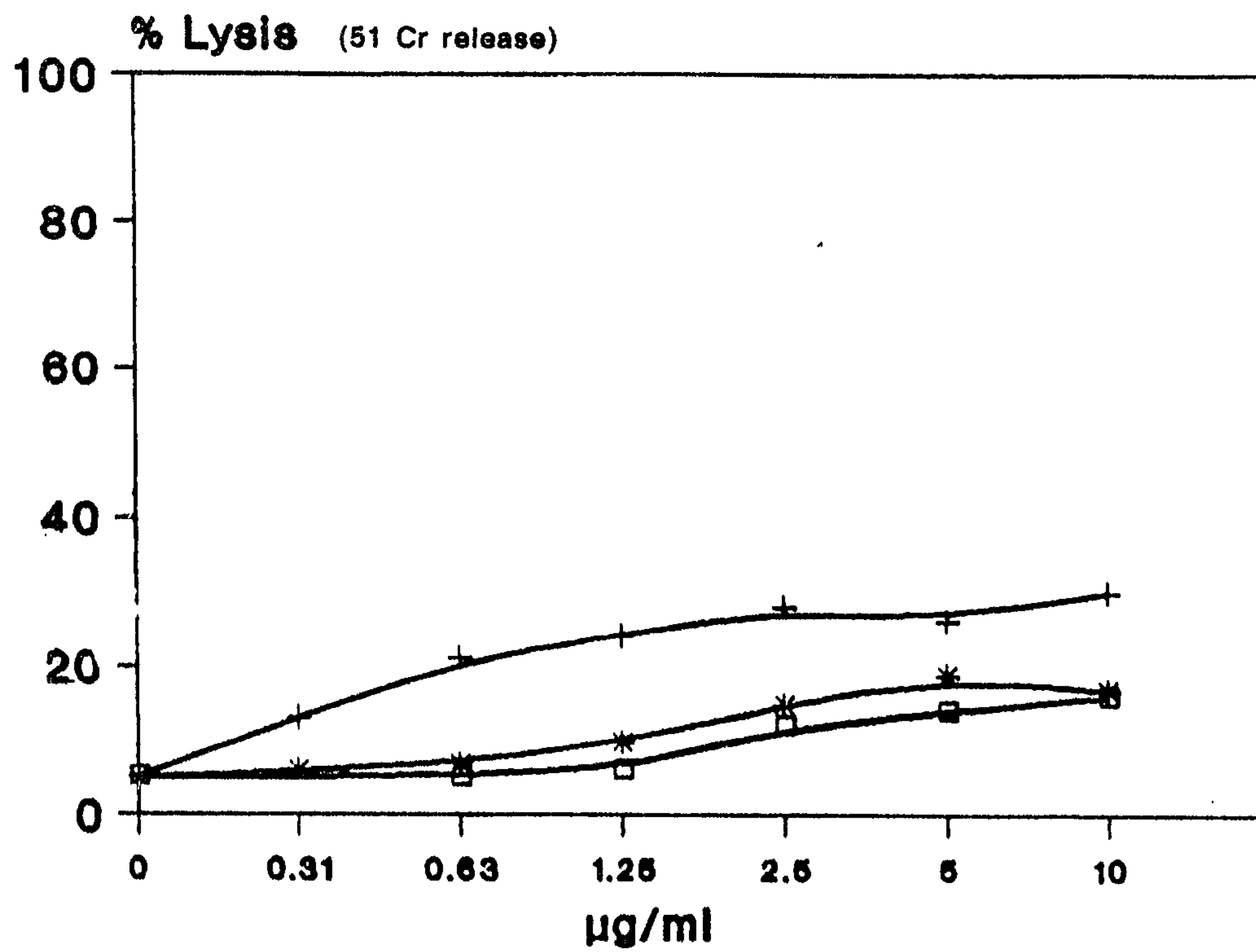
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Figure 32:

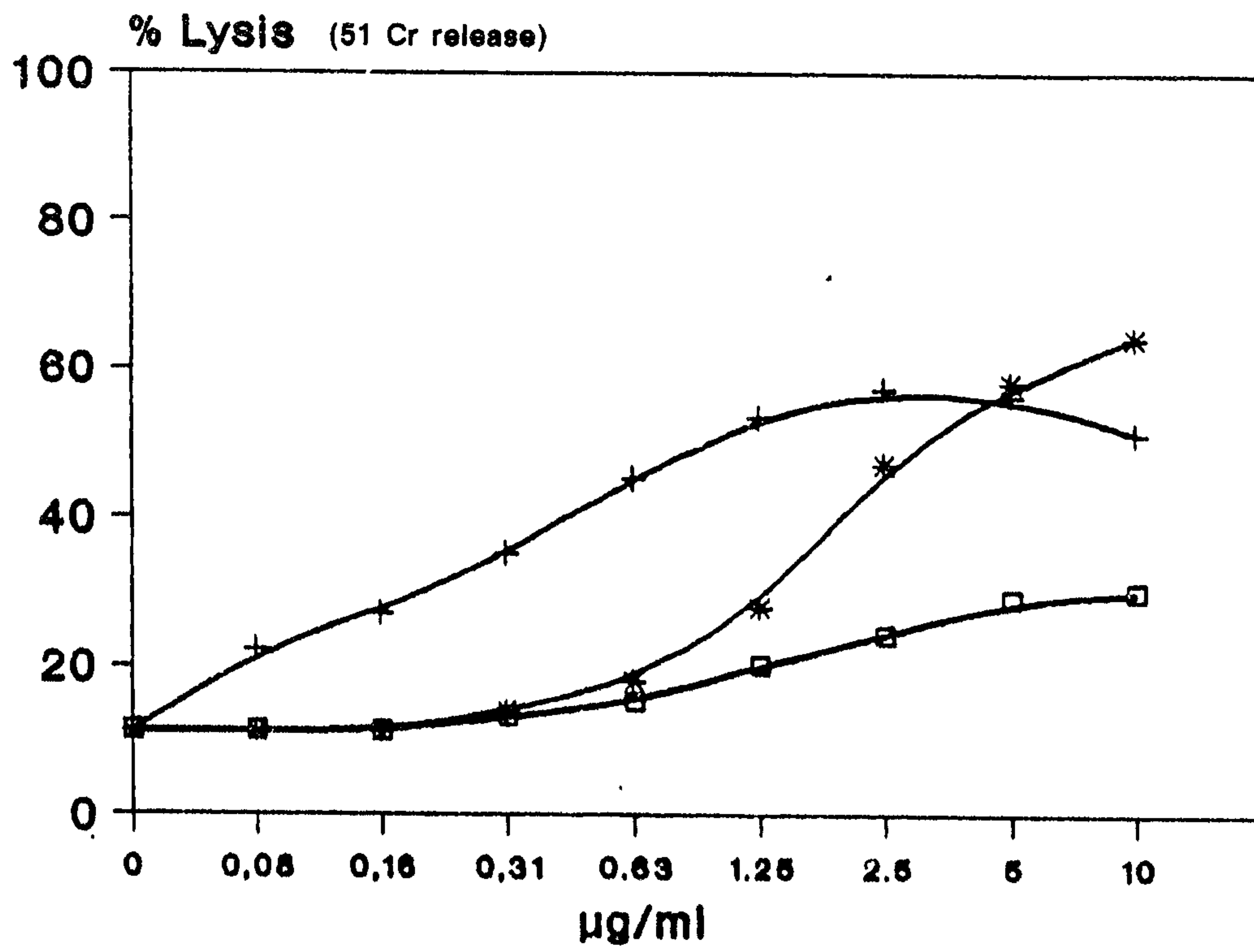
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Figure 33:

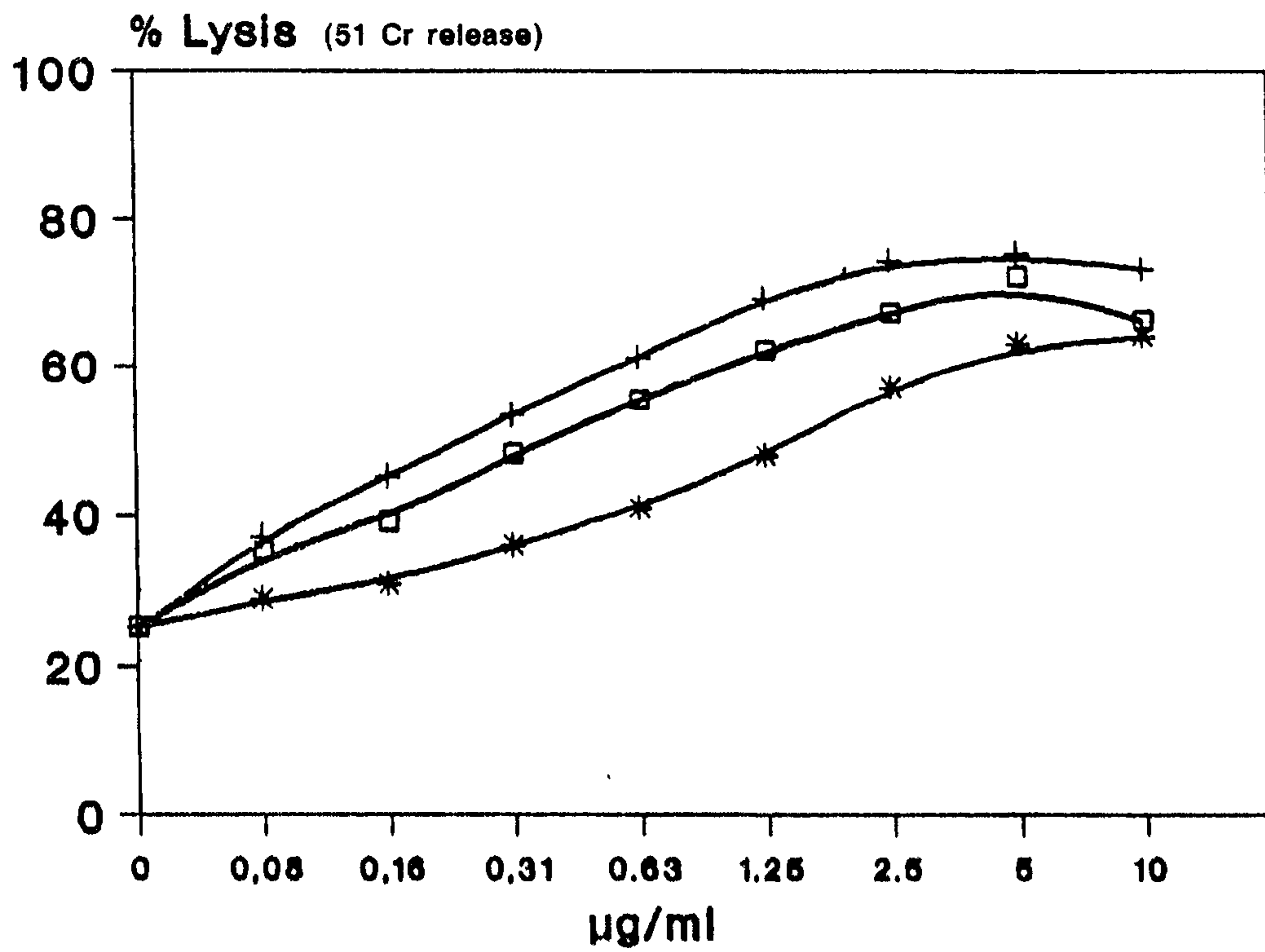
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Figure 34:

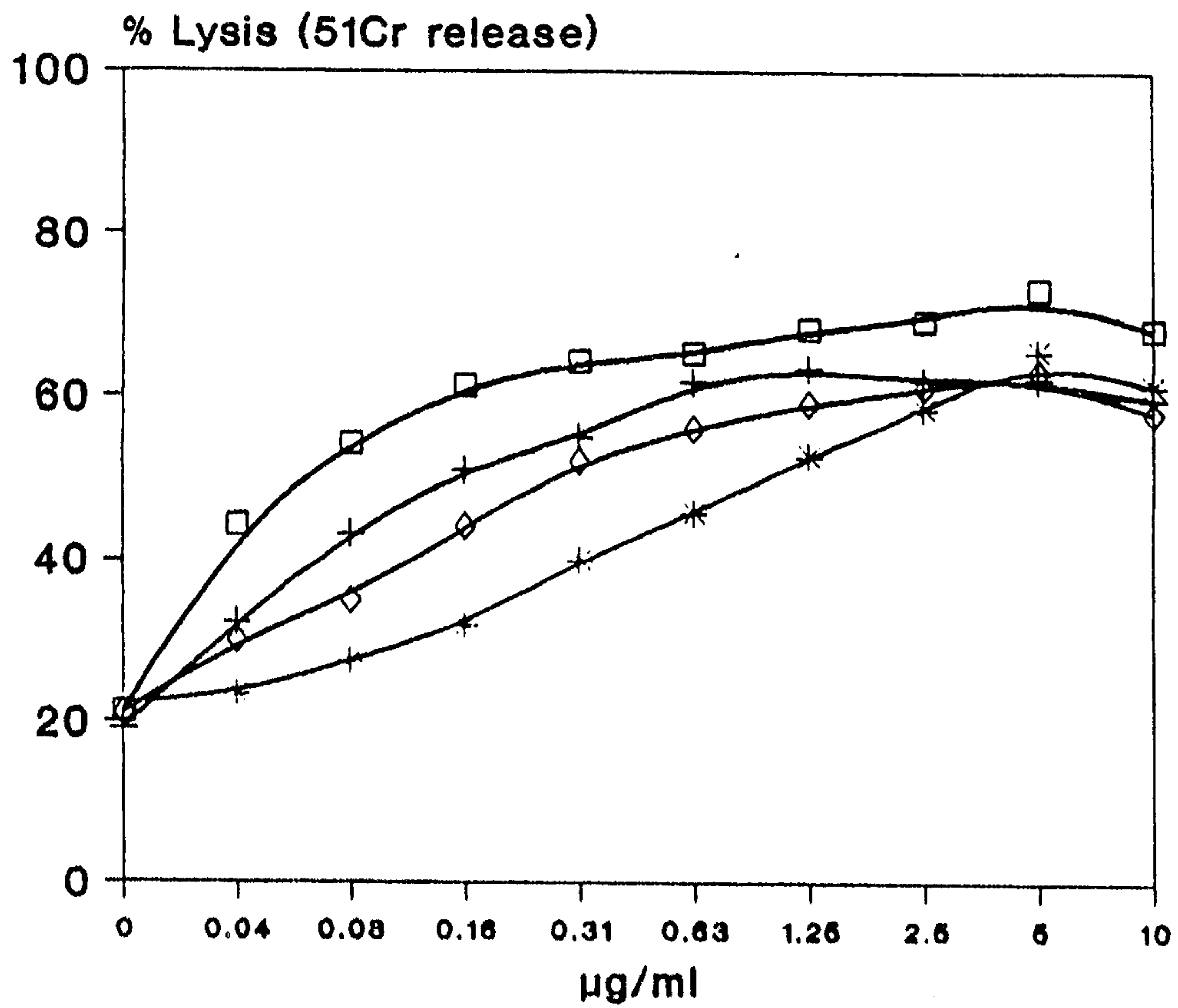
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Figure 35:

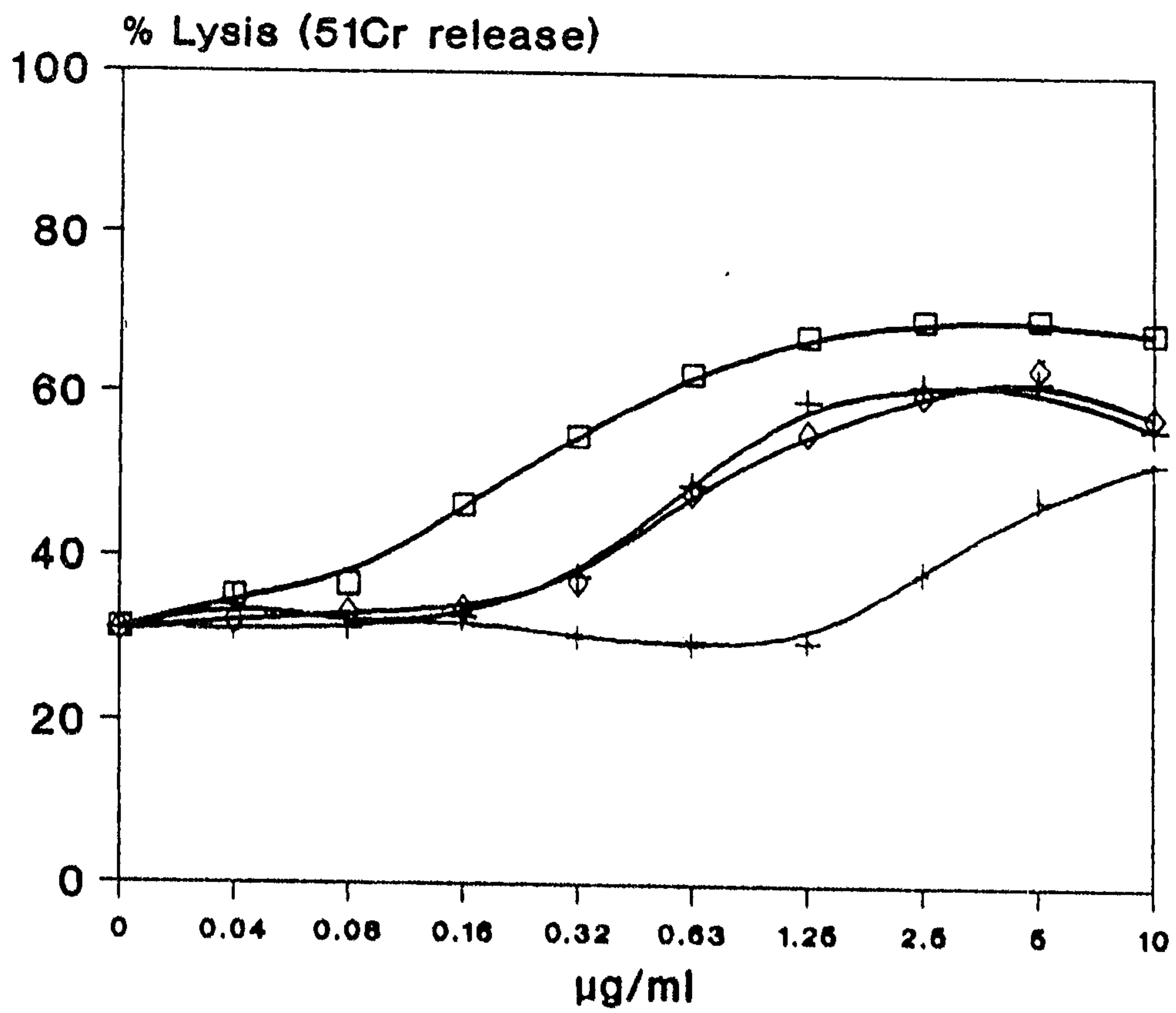
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Figure 36:

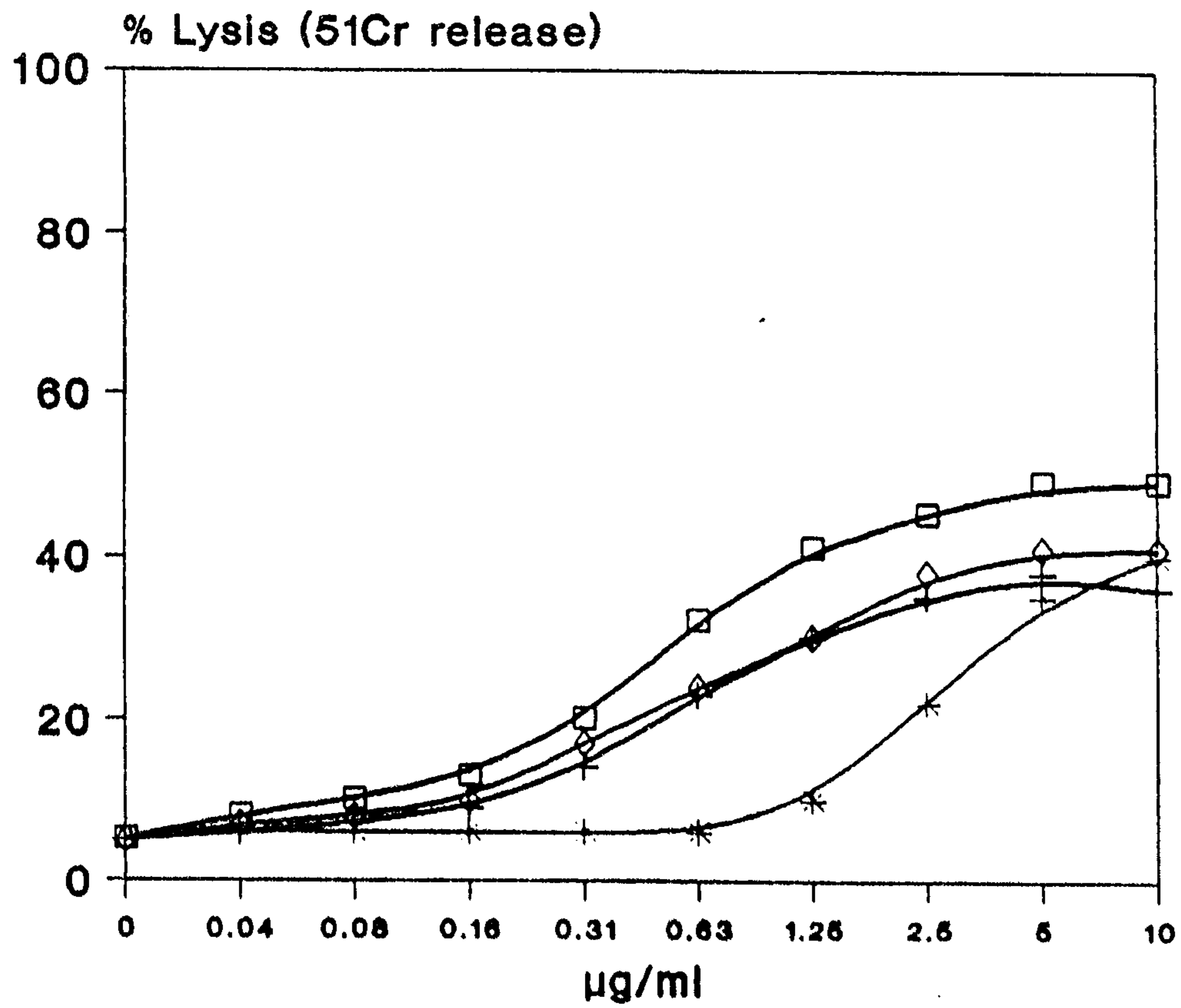
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Figure 37:

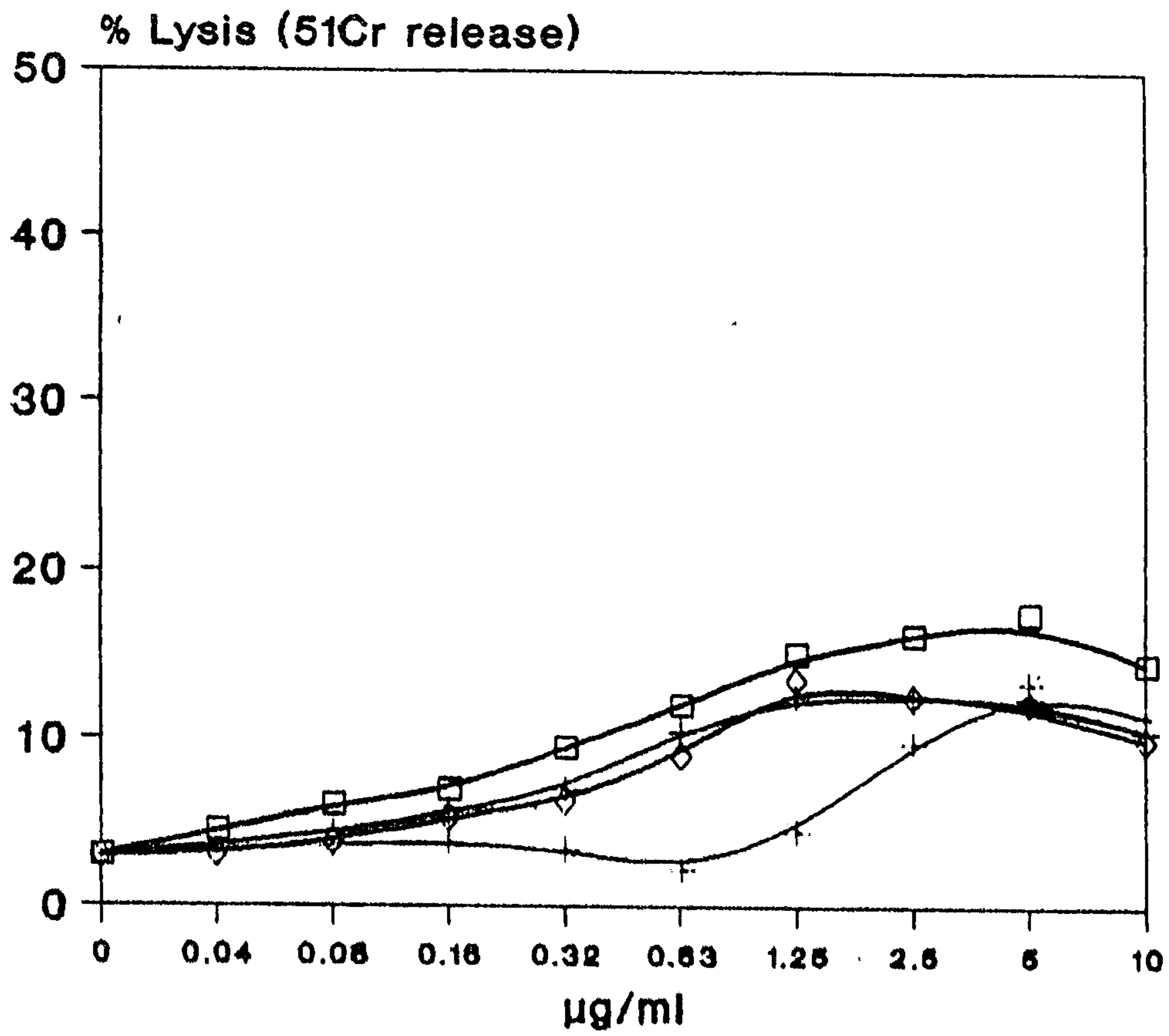
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Figure 38:

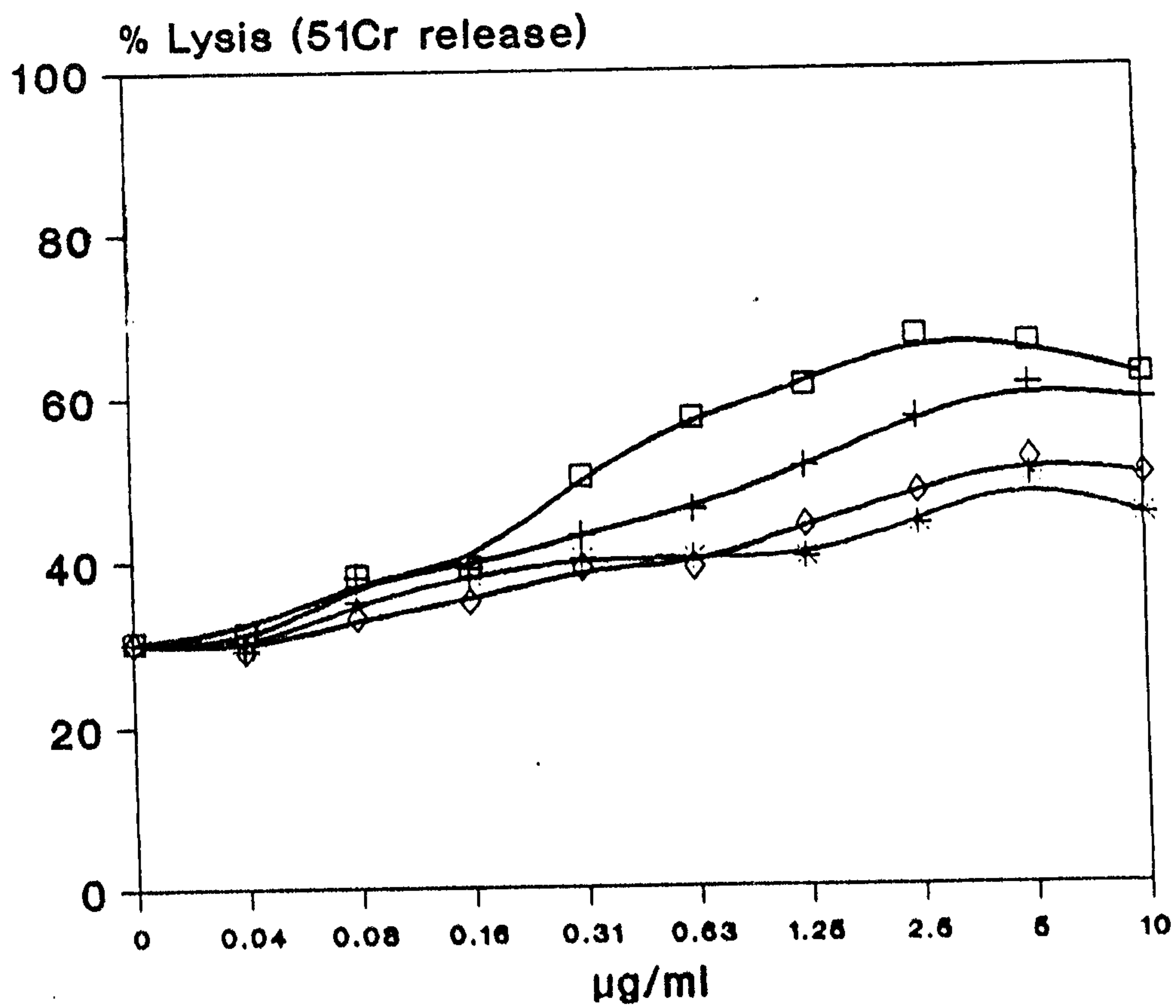
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Figure 39:

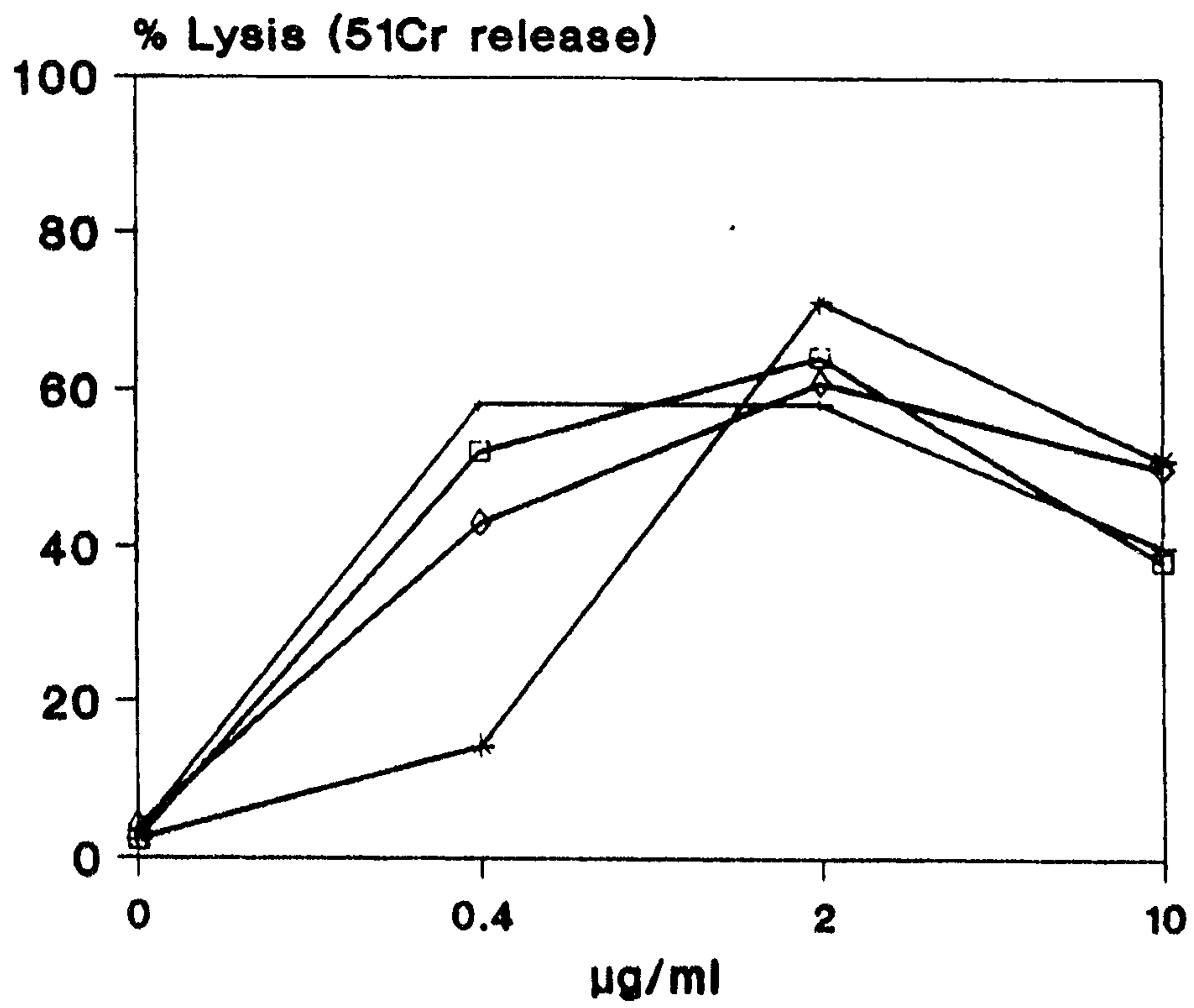
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Figure 40:

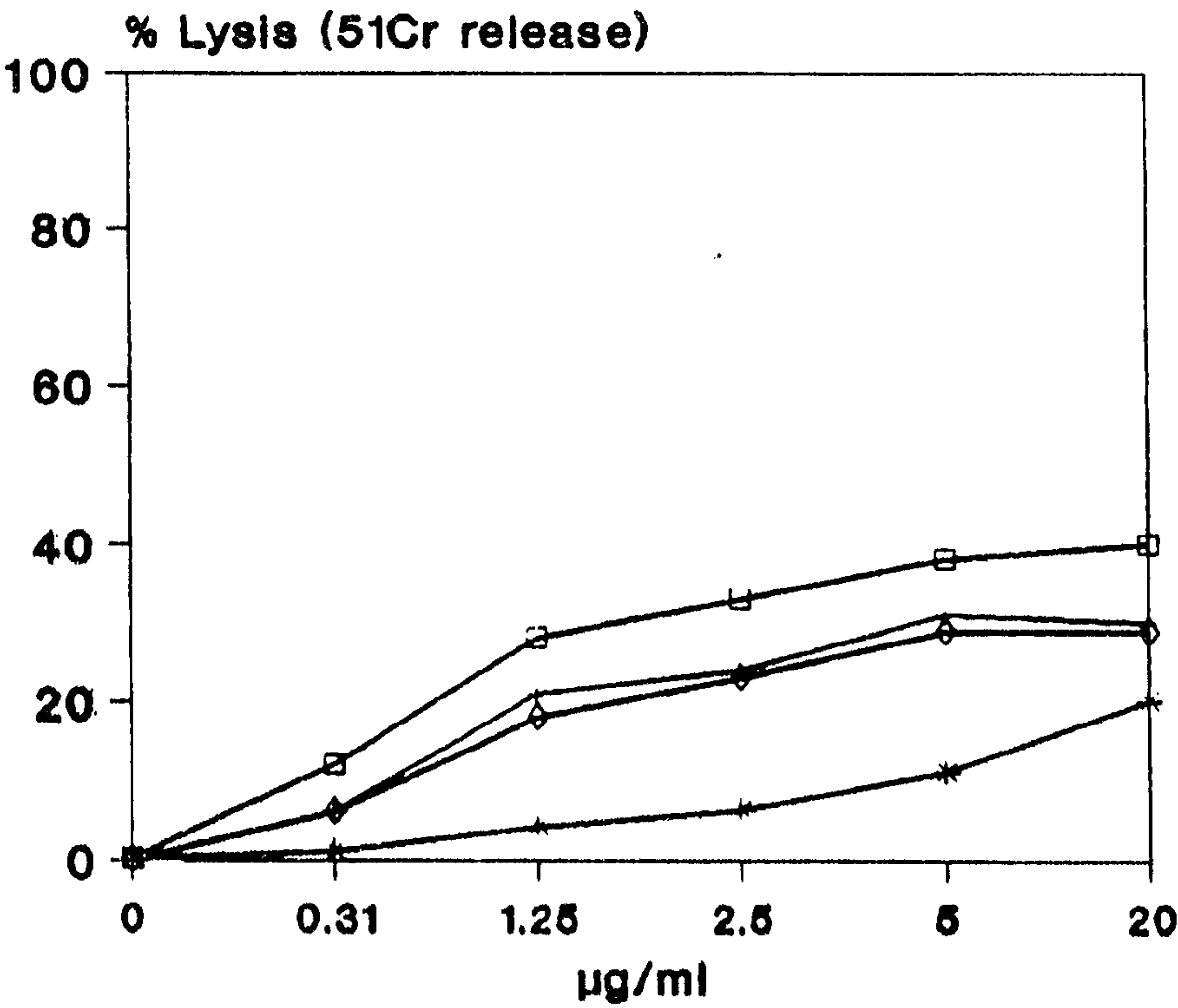
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Figure 41:

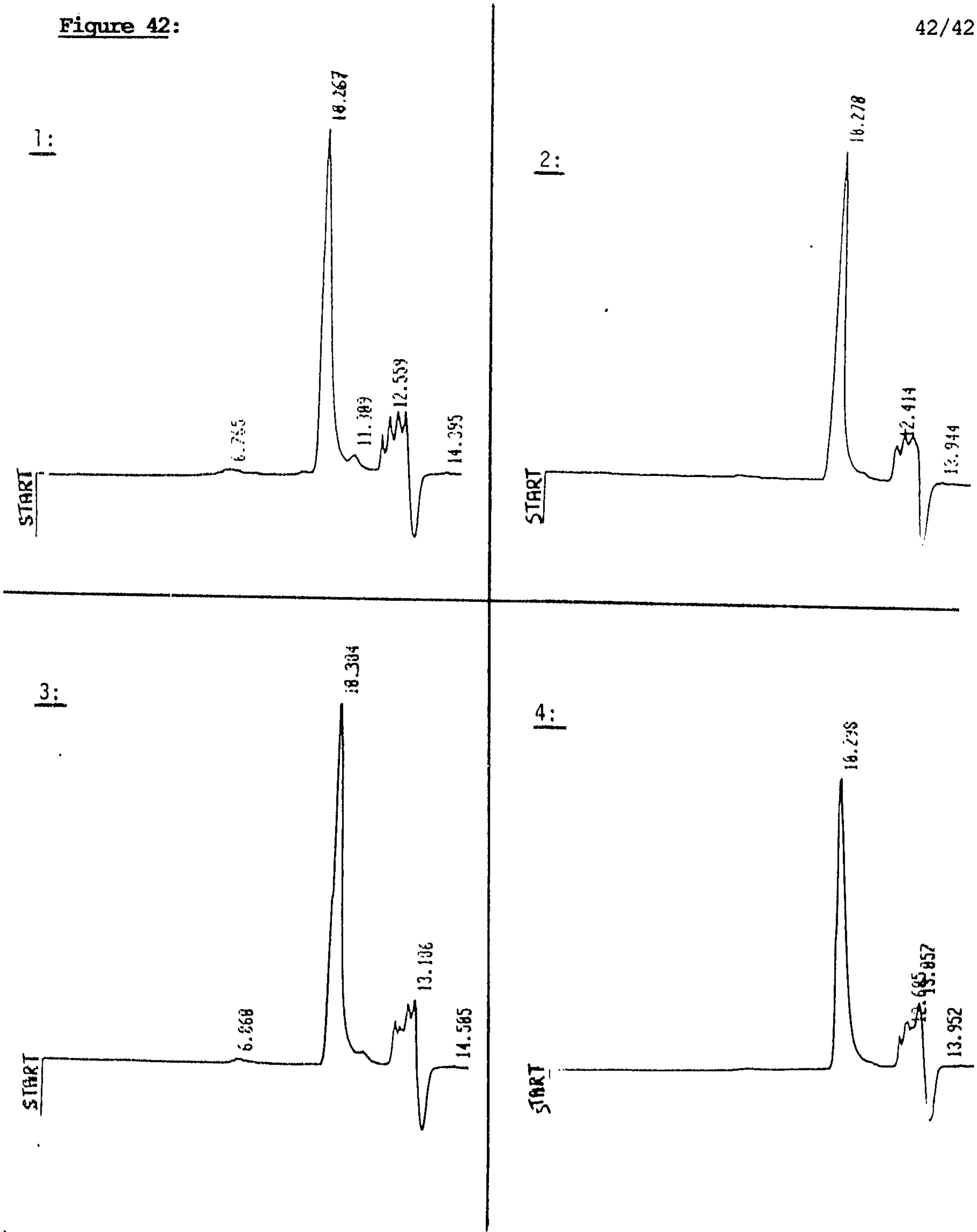
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Figure 42:

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