



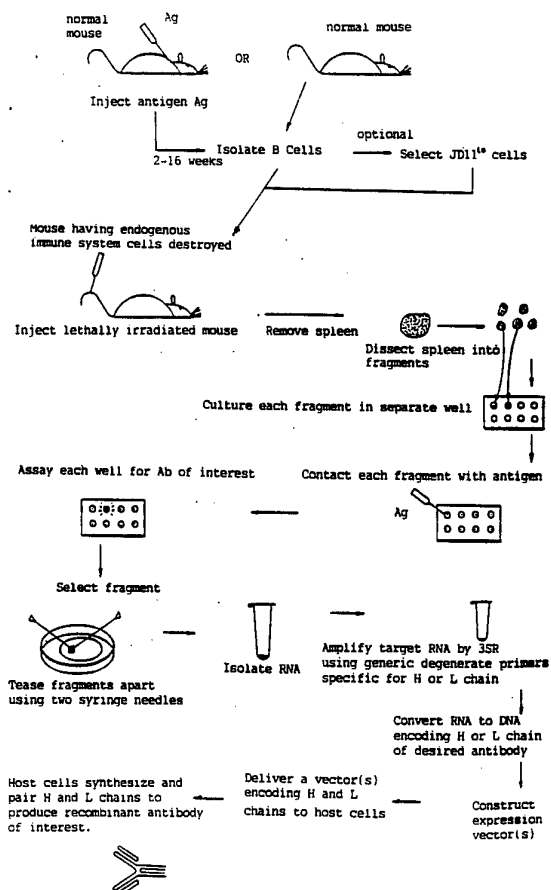
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(54) Title: PRODUCTION OF MONOCLONAL RECOMBINANT ANTIBODIES WITHOUT THE USE OF HYBRIDOMAS BY *IN VITRO* SPLEEN FRAGMENT CULTURE COMBINED WITH ISOTHERMAL SELF-SUSTAINED SEQUENCE REPLICATION OF RNA

(57) Abstract

The invention is directed to a method for producing a recombinant protein having a preselected binding affinity and specificity for a given antigen. Generally, the method combines spleen fragment culture for the *in vitro* somatic mutation of B cells in response to antigen, followed by preferential amplification of RNA encoding heavy and light chains of desired antibodies produced by those B cells. The amplified RNA is then converted to DNA and incorporated into expression vectors; transfected host cells then express the antibody chains or a single chain antibody having preselected characteristics. Also disclosed are generic degenerate primer pools for the efficient amplification of RNA encoding mouse IgG.



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PRODUCTION OF MONOCLONAL RECOMBINANT ANTIBODIES WITHOUT
THE USE OF HYBRIDOMAS BY IN VITRO SPLEEN FRAGMENT CULTURE
COMBINED WITH ISOTHERMAL SELF-SUSTAINED SEQUENCE
5 REPLICATION OF RNA

This is a continuation of US patent application serial
number 07/978,835, filed November 19, 1992.

10 Portions of the research described in this application
were supported by a grant from the National Institutes of
Health, and the U.S. government may have certain rights
in this invention.

15 Field of the Invention

The present invention relates to methods for the
production of recombinant monoclonal antibodies. The
methods of the instant invention are based on in vitro
20 spleen fragment culture of B cells which produce
monoclonal antibodies combined with the technique known
as isothermal self-sustained sequence replication (3SR),
which amplifies RNA encoding the antibodies of interest.
The invention also relates to generic degenerate primer
25 pools for use in replication of RNA encoding monoclonal
antibodies of interest.

Background of the invention

30 Monoclonal antibodies are used in therapy, diagnostics,
and basic research. For in vivo therapy, monoclonal
antibodies are directed against a toxin or a tumor
surface antigen to mount the patient's immune response
against the targeted toxin or pathogenic cell.
35 Alternatively, a monoclonal antibody may be conjugated
with a toxin, thereby directing the toxic action to the
tumor cell. Ex vivo, or extracorporeal immuno-therapy

involves the removal of the patient's bone marrow or blood and isolation of a selected cell population by means of binding a predetermined antigen on the cells' surface with a monoclonal antibody against that cell surface antigen. The monoclonal antibody may be chemically bound to magnetic beads which facilitate the removal of the cells bound to the antibody. Depending on the type of therapy being administered, the isolated cells may be discarded or they may be returned to the patient at a later date. Isolation of hemopoietic cells also offers the possibility of genetic manipulation of the patient's cells to correct a genetic defect or to expand a desired population of hemopoietic cells, which are thereafter returned to the patient. Immunodiagnostic tests are based on the generation of a signal proportional to the extent of binding of a monoclonal antibody to a specific substance (antigen) in a patient's blood or tissue.

Immunotherapy and reproducible immunodiagnostic tests require antibodies of standardized specificity and affinity for the antigen of interest. A significant step toward this goal was achieved with the development of hybridoma technology that allows the growth of clonal populations of cells secreting monoclonal antibodies with a defined specificity (Köhler, G. and Milstein, C., 1975, Nature 256:495-497). In this technique an antibody-secreting B-cell, isolated from an immunized animal, is fused with an immortalized myeloma cell. The products of this fusion are called hybridoma cells and are the source of monoclonal antibodies which currently provides the most reproducible source of antibodies for immuno-therapy and immunodiagnostics.

Production of hybridoma cell lines producing monoclonal antibodies of a desired specificity requires multiple

experimental steps which may extend over a significant time period. The immunization, screening and hybridoma production stages all pose problematic challenges in which costs increase in proportion to the amount of time and effort needed. Additional difficulties are at times encountered even after a monoclonal hybridoma cell line is identified and clonally selected. An expanded population of hybridoma cells can lose its ability to produce a specific monoclonal antibody after prolonged growth in culture. This propensity to lose antibody expression is also observed in hybridoma cell lines that have been frozen and stored after clonal selection. Thus, the longevity of valuable hybridoma cell lines is not predictable.

One approach to address this longevity problem has been to clone the light and heavy chains for a specific monoclonal antibody from the hybridoma mRNA pool and then to employ the polymerase chain reaction (PCR) to clone and express the variable domains of light and heavy chains of monoclonal antibodies in bacterial or mammalian cell lines (Orlandi, R., et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:3833-3837; Sastry, L., et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:5728-5732; Larrick, J.W., et al., (1989) Bio/Technology 7:934-938; Ward, E.S., et al., (1989) Nature 341:544-546). In so doing, a stable supply of a specific monoclonal antibody might be obtained if the host cell clone is sufficiently stable.

However, several factors suggest that this PCR/cloning approach may not always prove satisfactory. First, a hybridoma cell line must be identified and established in order to serve as a target for the PCR/cloning steps. As indicated above, this step may prove to be problematic. Second, the use of reverse transcriptase (RT)/PCR technique (Kawasaki, E.S., 1990, In: PCR Protocols. Eds.

M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White pp. 21-27) to clone light and heavy chain mRNA from a hybridoma cell line producing a monoclonal antibody of interest has frequently proven to be problematical. This RT/PCR failure has been attributed to the complication of non-productive immunoglobulin rearrangements which increase the complexity of the amplification-generated cDNA library. In turn, this significantly increases the likelihood of missing clones containing the productive immunoglobulin rearrangements.

In an effort to recover the originally paired V_H and V_L genes expressing the desired antibody, as well as to test novel synthetic V gene combinations, a bacteriophage display approach has been used. Antibodies engineered as a single chain or as heterodimeric Fab constructs have been displayed on the surface of a filamentous phage as a fusion of the phage pIII protein (McCafferty, J., et al., Nature 348:552-554). Fusions of the phage pVIII protein also have been used to display antibody fragments on the surface of the filamentous phage (Chang, C.N., et al., (1991) J Immunology 147:3610-3614; Huse, W.E., (1991) In: Borrebaeck, D.A.K. (ed.), Antibody Engineering. A Practical Approach., W.H. Freeman & Company, N.Y., p.103; Kang, A.S., et al., (1991) PNAS, USA, 88:11120-11123). Binding of the displayed antibody on the surface of the phage with antigen can be used as a way to recover antibodies composed of the original or novel V_H and V_L gene pairings. However, because each phage contains only one such V_H and V_L pairing, the frequency of the occurrence of any single V_H and V_L pairing is less than $1/10^8$. Consequently, this approach requires the construction of phage display libraries of 10^{11} copies to provide sufficient copies of any specific V_H and V_L combination for detection. In order to recover antibodies of especially high affinity or unique

specificity, the size of a phage display library may have to be even greater than 10^{11} copies.

How does an animal's immune system overcome these innate challenges to develop antibodies of the necessary specificity and high affinity? The differentiation of B cells in vivo and in vitro is described in Cell 59:1049-1059, 1989, Linton, et al. In vivo, immunization (exposure to foreign antigen) induces both a primary antibody response mounted by primary B cells and generation of T and secondary B cells which increase in specificity for the antigen and enable the organism to mount a more vigorous response upon additional exposure to the same antigen.

In order for the immune system to respond to the enormous spectrum of different antigens in the environment, it must be capable of producing antibodies with tremendous diversity. This problem is solved in part by the ability of immunoglobulin genes to rearrange the multiple forms of the four gene regions, V (variable), D (diversity), J (joining), and C (constant), prior to transcription and translation. Additional diversity is attained by the association of the heavy chain with one of many types of light chains. A primary antibody is composed of a light chain and a heavy chain, which are connected by a disulfide bridge to form a light/heavy chain complex; this complex is associated with other light/heavy chain complexes in either dimeric or pentameric forms.

Secondary B cells differ from primary B cells with respect to the variable regions which dominate a response. For each isotype of immunoglobulin (e.g., IgM, IgG, IgD), there are families of variable gene cassettes from which a single variable gene is selected in response to an antigen. Cells which carry an appropriate gene

cassette are known as "responsive B cells" for that particular antigen, even if they have not been previously exposed to that particular antigen, i.e. the responsive B cells have been pre-programmed to respond to the general three-dimensional configuration and physico-chemical attributes of the antigen of interest. Secondary B cells differ from primary B cells in their propensity to accumulate somatic mutations in the variable region to produce antibodies with substantially higher affinities for the antigen than those produced by primary B cells.

Clearly, it would be advantageous to be able to (1) isolate and identify a secondary B cell producing an antibody of desired affinity and specificity, in order to (2) isolate and clone the genetic sequence encoding that antibody. Once the genetic sequence is cloned, it is possible to use genetic engineering to produce the antibody of interest in the form of a recombinant protein. Considerable progress has been made towards achieving the first step via in vitro spleen fragment culture (Linton, et al., supra). However, it remains problematic to isolate and clone the immunoglobulin gene of interest because the identified B cells are expected to contain non-productive immunoglobulin gene rearrangements as well as the gene of interest. Therefore, attempts to amplify the gene of interest directly from DNA by PCR or RT/PCR, using primers encoding portions of the gene of interest, are complicated by the concomitant amplification of other productive and non-productive immunoglobulin gene rearrangements. The resulting amplified DNA would be expected to contain a complex mixture of the gene of interest and other genes. This situation would necessitate extensive screening of proteins expressed from the amplified DNA in order to identify a clone expressing an antibody of the desired specificity and

affinity. Moreover, there would be a low expectation of success from this approach.

What is needed is a means to selectively amplify nucleotide sequences encoding only the desired H and L chains.

Summary of the Invention

The invention is directed to methods for obtaining an amplified RNA encoding a predetermined chain of a desired antibody against a specific antigen. Briefly, the methods involve the steps of

(1) isolating B cells from one animal; optionally, J11D¹⁰ cells may be selected,

(2) injecting the B cells into a second animal,

(3) allowing the B cells to colonize the spleen of the second animal,

(4) culturing spleen fragments from the second animal,

(5) stimulating the spleen fragments with antigen,

(6) allowing B cells to proliferate and produce antibody in the cultures,

(7) assaying the cultures for a desired antibody,

(8) extracting total RNA from the fragment in an identified positive culture, and

(9) amplifying target RNA encoding the heavy or light chain of the desired antibody using 3SR in conjunction with a generic degenerate primer pool.

The methods of the invention can be extended to produce a desired recombinant monoclonal antibody by converting RNA encoding the heavy chain and RNA encoding the light chain to cDNA. The cDNA is converted into double-stranded DNA product by primer extension or RT/PCR and is then incorporated into one or two expression vectors, which are transfected into host cells. The host cells synthesize the recombinant monoclonal antibody possessing

the desired specificity and affinity for the antigen as identified in previous assays of the spleen cultures.

5 The invention is also directed to a method for constructing a generic degenerate primer pool for the efficient amplification of RNA encoding an immunoglobulin heavy or light chain using 3SR.

10 The invention is also directed to degenerate primer pools generic for mouse immunoglobulin light and heavy chains.

Definitions

15 For the purpose of construing the intended meaning herein, the following terms are defined as follows:

20 The term "B cell" encompasses the entire category of cells which lead to antibody production. Thus, "B cell" refers to a mammalian cell of hemopoietic origin whose progeny produce antibodies. The term "B cell" also includes stem cells or precursor cells which have the capacity to proliferate to produce progeny which ultimately produce antibodies. Herein, the term "B cell" also includes "memory B cells" or "secondary B cells" 25 that have been generated as the result of a previous immunization.

30 Herein the term "antigen" refers to a molecule which elicits an immune response in the form of antibodies produced by B cells. The antigenic molecule may be a protein or other molecule of biological or environmental origin. For instance, antibodies may be produced against a protein, a nucleic acid, or a steroidal hormone when B cells are stimulated by the specific molecule.

35

The term "responsive B cell" refers to a B cell which

contains an immunoglobulin gene rearrangement which enables the B cell to produce an antibody against a specific antigen. A responsive B cell may be a precursor cell which contains a gene cassette encoding an antibody of low specificity for the general three dimensional configuration and physico-chemical properties of the specific antigen. Later in differentiation, a responsive B cell may contain an immunoglobulin gene rearrangement which encodes an antibody of greater specificity and affinity for the specific antigen. The above defined term "B cell" is understood to include "responsive B cells".

The term "antibody" minimally refers to an immunoglobulin having at least one light and one heavy chain (Fab), or at least two light chains and two heavy chains, each chain having a variable and possibly a constant region. The antibody may be an IgG, an IgA, an IgE, an IgM, or an IgD.

The term "monoclonal antibodies" refers to antibodies which are identical to each other because they have been produced by cells arising from a single cell clone expressing defined H and L chain genes encoding the antibodies.

The term "recombinant antibody" refers to an antibody encoded on an expression vector contained in host cells and expressed as a protein by the host cells. A recombinant antibody may be in the form of heavy chains and light chains bound by di-sulfide bonds, just as a naturally occurring antibody. Alternatively, a recombinant antibody may be a single-chain antibody construct which has the capability to bind a specific antigen.

The term "heterogeneous RNA" refers to RNA which encodes many different cellular proteins. Thus the term "heterogeneous RNA" encompasses total RNA extracted from a culture of cells, as well as poly(A)+ RNA isolated from total RNA extract.

The term "target RNA" refers to RNA which encodes a single protein.

The acronym "3SR" refers to isothermal self-sustained sequence replication. The term "isothermal self-sustained sequence replication" refers to a process by which a target RNA is amplified in a process which essentially mimics the way in which retroviral RNA is amplified in nature. Alternative terms are 3SR and TAS. Generally the process makes use of three different enzyme activities: reverse transcriptase, RNA polymerase, and RNaseH. The word "isothermal" refers to the fact that the process is carried out at a single or narrow temperature range below about 95°C so that double-stranded DNA is not denatured, and therefore is not amplified. This feature makes the process specific for the amplification of RNA rather than double-stranded or genomic DNA. During the process, the temperature may be varied over any range at which all the necessary enzymes function. Thus, the word "isothermal" is not intended to abrogate variations in the temperature range below about 95°C.

The term "degenerate primer pool" refers to a collection of primer sequences in which many or all possible variations in the target sequence are represented.

The term "generic degenerate primer pool" refers to a degenerate primer pool in which the variations are limited to those sequence substitutions represented in a

catalogue of sequences known for a specified immunoglobulin chain. Thus, for instance, a degenerate primer pool generic for mouse IgG light chain would contain sequences encoding a 3' constant region as well as all possible sequences known for 5' variable regions of all known mouse IgG families such as lambda and kappa.

The term "consisting essentially of", in reference to an amplified target RNA, means that the product contains the amplified 3SR product RNA in overwhelming proportion to any other amplified RNA or DNA sequences derived from non-target RNA which may have been present in the starting material.

Brief Description of Drawings

Figure 1 depicts the major steps in producing a monoclonal recombinant antibody using the methods of the invention.

Figure 2 defines the sequences of oligonucleotide primers referred to herein by the number in the far left column of Figure 2.

~~Figure 3 shows Northern (A) and Southern (B) blot analyses of 3SR and PCR amplification products. (A) 3SR RNA products using heavy chain leader (HC-L) and light chain variable (LC-V) 5'end primers. (B) The 3SR RNA products were converted to c-DNA. LC=light chain; HC=heavy chain; M=size markers of MspI digested pBR322 fragments; S=anti-PC specific 5'end primers; G_v=generic variable region 5'end primers; G_L=generic leader region 5'end primers~~

³
Figure ~~4~~ is a scheme for the construction of plasmid pT15-110 which encodes a single chain (sc) antibody form

of the anti-PC antibody. MBS=metal binding site; TAG=c-myc tag; LC=light chain; HC=heavy chain.

~~Figure 5 shows nucleotide and amino acid sequences of the four heavy (4A) and light (4B) chain groups (G₁₋₄) cloned from spleen fragment culture D₁A₆ (anti-E2). The CDR regions are noted in the heavy and light chain groups by overlining the appropriate sequences. The heavy chain sequences include the 5'end variable region primers (Figure 2) and the light chain sequences include only the last three nucleotides of the 5'end variable region primers (Figure 2).~~

Detailed Description

The general concept of the invention is outlined in Figure 1 as follows:

B cells are isolated from a donor mammal that has been previously immunized with a specific antigen. [Optionally, a non-immunogenized mammal may be used as the donor. Optionally, B cells may be further selected for precursor status by their lack of memory B cell markers.]

Isolated B cells are injected into a recipient mammal without viable endogenous immune system cells. Optionally, the recipient may have been previously immunized with all or part of the antigen to promote T cell helper function. The injected B cells are allowed to colonize the recipient's spleen.

Spleen of the recipient is removed and dissected, each fragment being placed in a separate culture well.

Spleen fragments are maintained in culture and stimulated

with antigen. Certain fragments contain responsive B cells which will proliferate to produce progeny which synthesize antibody against the antigen.

5 Each culture is assayed for an antibody of interest, based on desired affinity and specificity for the antigen. A culture containing a positive fragment is identified.

10 The positive fragment is extracted for total RNA.

RNA from the positive fragment is subjected to 3SR amplification of the RNA encoding the antibody of interest. Generic degenerate primer pools are provided
15 herein for the amplification of RNA encoding mouse immunoglobulin light and heavy chains.

RNA encoding the antibody of interest is converted into a double-stranded cDNA.

20 Double stranded cDNA encoding the antibody of interest is incorporated into an expression vector.

The expression vector is transfected into a host cell.

25 The desired antibody is expressed by the host cell and isolated.

Preferably, a donor animal such as a mouse is immunized
30 with an antigen of interest such as estradiol. Injection of antigen will stimulate the proliferation and differentiation of B cells which produce a primary antibody against the antigen as well as memory B cells. Alternatively, a naive animal, i.e. one which has not
35 been injected with antigen, may be a donor of B cells since it is expected that certain precursor B cells

possess gene rearrangements encoding antibodies of low specificity for a large repertoire of antigenic characteristics, including those of the antigen of interest.

5

After a period of 2 - 16 weeks, in the case of the immunized donor, B cells are isolated from the donor animal's bone marrow, peripheral blood, or spleen. Alternatively, B cells may be isolated immediately from a naive donor. The isolated B cells may be subjected to a further selection process whereby cells which exhibit low binding capacity for J11D monoclonal antibody [J11D^{lo} cells] are selected for injection (Litton, et al., Cell 59:1049-1059, 1989). The J11D^{lo} cell population is considered to be enriched for secondary B cells, which are expected to produce antibodies of higher affinity and specificity for the antigen of interest.

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Isolated B cells are injected into a recipient animal which does not possess viable endogenous immune system cells. The recipient animal may have been "lethally irradiated" such that essentially all its immune system cells have been destroyed. The term "lethally irradiated" refers to the killing of immune system cells and does not refer to killing of the animal itself. However, without sterile isolation or immune system replenishment the lethally irradiated animal would soon succumb to infection. Alternatively, the recipient animal, for instance an SCID mouse, may be genetically deprived of an immune system. In either case, the spleen of the recipient animal is denuded of endogenous B cells and is open for colonization by injected B cells.

35

The concentration of injected B cells is calculated so that, on average, only one responsive B cell will colonize a predefined segment volume of the recipient

animal's spleen. In the case of a mouse recipient, the concentration calculation is described in Linton, et al, (supra).

5 The recipient animal is maintained for sufficient time to allow the injected B cells to colonize the spleen. Preferably, the recipient animal is also immunized with antigen to stimulate helper T-cell function, which facilitates B cell proliferation and differentiation. If
10 an immune deficient recipient is used, the transfer of antigen stimulated helper T cells is needed to facilitate B cell proliferation and differentiation.

The spleen is then removed from the recipient animal and
15 dissected into fragments, the size of the fragments being chosen so that each fragment contains, on the average, only one responsive B cell. In the mouse system described in Linton, et al., (supra), the optimum fragment size is about 1 mm³.

20 Each spleen fragment is placed in a separate culture well, typically in 96 well plates. The fragments are maintained in culture as described in Example 1 below.

25 To each culture well, an aliquot of the antigen of interest is added. In any given spleen fragment where there is a responsive B cell for the antigen, the responsive B cell is expected to proliferate and differentiate to produce multiple cells which synthesize
30 antibodies of increased specificity and affinity for the antigen of interest.

The medium of each culture well is assayed for affinity and specificity for the antigen of interest.

35 A positive well is identified which contains a spleen

fragment having B cells producing the antibody of interest. The spleen fragment technique for obtaining monoclonal antibodies has several advantages over hybridoma technology. The time and expense involved in production and screening of spleen fragments is expected to be less than the time and expense involved in producing hybridomas as described above. Additionally, the spectrum of antibody products may be much greater using the spleen fragment technique.

From the identified spleen fragment, total RNA is extracted. Optionally, poly(A)+ RNA may be isolated from the total RNA. In either case, the resulting heterogeneous RNA encodes many proteins synthesized by the B cells. The target RNA species, which encode light and heavy chains for the antibody of interest, are expected to represent only a portion of the total RNA or the poly(A)+ RNA extracted therefrom.

The next task is to amplify the RNA which encodes the heavy and light chains of the antibody of interest. This task is made difficult by the inevitable presence of non-desired extraneous nucleotide sequences which have certain homologies to the RNA of interest but which, if amplified, will yield a non-productive genetic sequence. That is, amplified extraneous nucleotides would probably not encode a functioning antibody, and they certainly would not encode the previously identified antibody of interest.

The problem with amplification of non-productive gene rearrangements was recognized in early attempts by the present inventors to amplify the immunoglobulin gene of interest using polymerase chain reaction (PCR). PCR is based on cycles of denaturing doubled stranded DNA by temperatures above about 95°C to form single stranded

DNA, lowering the temperature to activate DNA polymerase which copies the DNA sequences complementary to the single strands, followed by again raising the temperature above about 95° C to separate the amplified double stranded DNA. It was recognized that double-stranded DNA should be left unamplified in order to increase the chances for amplification of the transcribed gene of interest without concomitant amplification of non-productive gene rearrangements. This suggested that statistically the chances of amplifying the gene of interest might be better out of the RNA pool rather than the DNA pool. However it was known that non-productive gene rearrangements also existed in the RNA pool, making the results from RNA amplification also unpredictable.

Fortuitously, it was found that amplification of RNA using 3SR in conjunction with generic degenerate primer pools could work to produce amplified RNA encoding the gene of interest. The basis for 3SR is described in PNAS 87:1874-1878, 1990, Guatelli, et al; J. Infect. Dis. 164:1075-81, 1991, Richman, et al; J. Infect. Dis. 164:1066-74, 1991, Gingeras, et al. For reviews, see also Fahy, et al, In: PCR Methods and Applications pp. 25-33, Cold Spring Harbor Laboratory Press, 1991; Gingeras and Kwoh, In: Praxis der Biotechnologie, In Vitro Amplification Techniques, pp. 403-429, 1992, Publ: Carl Hanser; Gingeras, et al., Ann. Biol. clin. 48:498-501, 1990.

Briefly, in 3SR, a continuous series of reverse transcription and RNA transcription steps replicates a nucleic acid target by means of cDNA intermediates. Two or three different enzymes work together in this system; typically a reverse transcriptase, an RNaseH, and an RNA polymerase are required. Alternatively, exogenous RNaseH (i.e. from E. coli) may be omitted when reaction

conditions are optimized (Fahy, et al., supra). 3SR can be conducted in a single pot at a single temperature, below 95°C, which allows the activity of the enzymes in the reaction. The temperature is chosen to optimize the activity of the specific enzymes. Currently, 3SR enzymes are optimally active in the temperature range of 37°C to 42°C. However, new enzymes are in development which may be optimally active at substantially higher temperatures, although the 3SR reaction will routinely be conducted below about 95°C.

The RNA of interest is targeted by a primer set including a specific 3' primer and a 5' primer pool. The 3' primer encodes a T7 promoter as well as antisense for the RNA of interest. Since the constant region of each type of immunoglobulin generally has a well defined single sequence, the 3' primer can be clearly defined. Primer sequences for the 3' region of mouse immunoglobulin heavy and light chains are listed in Figure 2 (91-267*, 91-268*, 92-004, 92-006, 92-002*).

Devising primers for the 5' end, however, is complicated by the great variety of sequences possible for the variable region. It is not predictable which immunoglobulin variable chain family is present in the antibody of interest. Therefore, a degenerate 5' primer pool, generic for all variable chain families of the animal species of interest, is designed to enable efficient amplification of the RNA of interest.

For illustration of the basic principles, the following describes the design and synthesis of a degenerate 5' primer pool generic for mouse variable region families. Degenerate primers were designed using the data base of Kabat et al. (In: Sequences of Proteins of Immunological Interest, 4th Edition (1987) U.S. Dept. Health and Human

Services), taking into account codon degeneracies for each amino acid in the conserved sequences of the leader and FR1 regions. The degenerate pool generic for the light chain is depicted in Figure 2 (sequences 92-099 to 92-102), for the heavy chain starting with the leader sequence in Figure 2 (sequences 92-107 and 92-108), and for the heavy chain starting with the coding region for the mature protein in Figure 2 (sequences 92-095 to 92-098 and 92-109 to 92-110).

Oligonucleotide primers were synthesized using an Applied Biosystems Incorporated DNA synthesizer using phosphoamidite chemistry. Parentheses in Figure 2 indicate a single degenerate site. Wherever nucleotide bases are indicated in parentheses in Figure 2, those bases should be added in equal proportion at that site to obtain a degenerate pool which is truly representative of all the possible variations at each site. It was recognized that, in order to increase the chances of amplification of the RNA of interest, it would be important to have the correct 5' primer present in the pool in proportion to the 3' primer.

However, it was also recognized that, in synthesizing degenerate sequences, often a base substitution is underrepresented at a certain site in spite of adding equal proportions of the nucleotides to the synthesizer pool at the appropriate point in the synthesis. To address this problem, equal representation of all possible substitutions at selected 3' sites of the 5' primers was forced according to the following method.

Each of the degenerate sequences was synthesized in a separate sub-pool. The following description of the synthesis of the degenerate pool for the light chain [Figure 2, sequences 92-099, 92-100, 92-101, and 92-102]

illustrates the principle for synthesis of the pool.

Briefly, the oligonucleotide synthesis of sub-pool 92-099 was begun by the addition starting at the 3' end of A,

then C, followed by C. Then A was specifically added at the degenerate site four nucleotides from the 3' end.

Oligonucleotide synthesis was continued with the addition of C followed by T. Then A and G were added to the reaction mix in 1:1 proportion for addition at the heterogeneous site 7 nucleotides from the 3' end.

Synthesis was continued with the specific addition of A followed by C. Then G, C, and A were added to the reaction mix in the proportion of 1:1:1 for addition at the heterogeneous site 10 nucleotides from the end.

Oligonucleotide synthesis was continued in this manner until degenerate sub-pool 92-099 was complete. Each degenerate sub-pool was synthesized in this manner, resulting in four separate primer sub-pools. Then the sub-pools were combined in equal proportion to form the light chain 5' degenerate primer pool generic for mouse light chain immunoglobulin. By this method, it was assured that the total pool would have an equal representation of A, T, C, and G at the position four nucleotides from the 3' end.

The rationale for forcing equal representation at the 3' end of the 5' primer ensures that exponential, not linear, increases are observed throughout the amplification period of the 3SR reaction.

Thus the degenerate primer pool generic for the mouse immunoglobulin light chain comprises a 5' primer pool having an equal proportion of I, II, III, and IV, wherein

I is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCA CCA

II is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCT CCA

III is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCC CCA

IV is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCG CCA

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wherein X₁=T or C, X₂=T or C, X₃=G or C, X₄=G, C, or T, X₅=A, C, or G, and X₆=A or G.

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The above described degenerate 5' primer pool must be combined with a 3' primer in order to allow 3SR amplification of the RNA of interest. Since the most common type of secondary antibody is IgG, it is preferred to use a 3' primer specific for the constant region of mouse IgG. Preferably, the degenerate primer pool for the mouse IgG light chain further comprises a 3' primer having the sequence 92-002* or 92-006 in Figure 2, said degenerate primer pool comprising one part of said 3' primer and four parts of said 5' primer pool. This proportion of 1:4, 3' pool:5' pool, facilitates the efficient amplification of the RNA of interest because it increases the chances that the appropriate 5' sequence will be present in proportion to the 3' sequence.

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A degenerate 5' primer pool generic for the mouse immunoglobulin heavy chain, coding from the leader sequence, may be constructed to contain an equal proportion of I and II, wherein

I is:

ATG X₁AX₂ TTX₃ X₄GG X₅TX₆ AX₇C TX₈G X₉TT

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II is:

ATG X₁₀AA TGX₁₁ AX₁₂C TGG GTX₁₃ X₁₄TX₁₅ CTC T

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wherein X₁ = G or A, X₂ = G or C, X₃ = G or C, X₄ = T or G, X₅ = T or C, X₆ = A or C, X₇ = A or G, X₈ = G or T, X₉ = G or A, X₁₀ = G or A, X₁₁ = G or C, X₁₂ = G or C, X₁₃ = C or T, X₁₄ = T or A, X₁₅ = T or C.

For 3SR amplification of mouse IgG heavy chain, the above 5' primer pool for the heavy chain leader sequence is combined with a 3' primer specific for mouse IgG heavy chain constant region. The 3' primer has the sequence 91-267*, 91-268*, or 92-004 in Figure 2. The total primer pool comprises one part of the 3' primer and two parts of the 5' primer pool.

A degenerate 5' primer pool generic for the mouse immunoglobulin heavy chain, coding from the mature amino acid sequence, may be constructed to contain an equal proportion of I, II, III, and IV, wherein

I is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCA GG

II is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCT GG

III is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCC GG

IV is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCG GG

wherein X₁ = G, C, or A, X₂ = G, C, or A, X₃ = A or G.

For 3SR amplification of mouse IgG heavy chain, the above 5' primer pool for the heavy chain mature sequence is combined with a 3' primer specific for mouse IgG heavy chain constant region. The 3' primer has the sequence 91-267*, 91-268*, or 92-004 in Figure 2. The total primer pool comprises one part of the 3' primer and four parts of the 5' primer pool.

Using the above described primer pools, target RNA's encoding the light chain and the heavy chain are amplified in two separate rounds or a single round of 3SR reaction.

Given the above description, it will be apparent to one of skill in the art that other techniques besides 3SR can be used to obtain amplified RNA. For instance, the technique known as RT/PCR may soon evolve to utilize temperatures which do not denature double-stranded DNA, and thus RT/PCR could be substituted for 3SR to preferentially amplify RNA. Another technique known as "Q Beta Replication" is based on a phage enzyme which replicates negative strand RNA into positive strand RNA, thus also providing preferential amplification of RNA.

For each amplified product RNA, complementary double stranded DNA is made using primer extension or RT/PCR.

Once the cDNA for each chain of the antibody of interest is obtained, there are several routes to choose for making the recombinant antibody.

Each double stranded DNA of the 3SR products can be inserted into a separate expression vector, both vectors can be transfected into a host cell such as E. coli, and the host cell will synthesize both immunoglobulin chains and cause them to associate properly via di-sulfide bonds to form a functioning antibody. Alternatively, a plasmid containing both light and heavy chain genes under the regulation of separate promoters may achieve expression of each of the antibody chains. In either case, with a single vector or multiple vectors, the two Ig chains can then be expressed by a single host cell. Alternatively, each vector can be transfected into a separate host cell, each of which produces a light or a heavy chain separately. The two immunoglobulin chains can then be isolated from the host cells and caused to form appropriate di-sulfide bonds by manipulating the pH and/or urea and salt content of the medium surrounding the proteins as described in U.S. Patent No 4,816,397.

In yet another alternative, portions of the sequences of the light and heavy chains can be linked to encode a single-chain antibody which is then expressed by the transfected host cell as described in Example 3 below.

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Thus, practicing the methods of the invention, and without the use of hybridoma technology, it is possible to obtain a recombinant monoclonal antibody of desired specificity and affinity for a predetermined antigen.

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Unlike cell suspension cultures, spleen fragment cultures retain sufficient germinal center architecture to enable memory B cell generation and somatic mutation in response to antigen. The invention is advantageous over hybridoma technology and phage display technology because the invention allows for greatly reduced screening.

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Moreover, the invention makes possible the preselection for: 1) specific isotypes, 2) antibodies with binding affinities of desired levels, and 3) defined specificity as determined by competitive analog screening. Using the methods of the invention, screening is performed on a relatively small library of antibodies which were recombined and paired by the innate processes of the responsive B cells in the germinal centers of the spleen fragments. Thereafter, the nucleotide sequences encoding predominantly the desired antibody chains are selectively amplified, cloned, and expressed.

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The following experiments are described by way of example to illustrate the methods of the invention and are not to be construed as limiting the scope of the invention.

EXAMPLE 1

Design and synthesis of primers specific for the anti-PC monoclonal antibody.

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T15 refers to an epitope of certain antibodies specific for the Pneumococcus coat protein (PC) which is found on

two hybridoma cell lines (R2-26 and R2-09) that produce anti-PC monoclonal antibodies. Nucleotide sequences for both light and heavy chains of T15 antibodies have been determined previously. The T15-specific primers were selected from published sequences for the V_k T15(S107) light chain rearrangement and V_H S107 T15 heavy chain. The sequences of the specific primer pair are listed in Figure 2 (heavy chain:92-001*/91-267*; light chain: 92-003*/92-002*). Oligonucleotide primers were synthesized with an Applied Biosystems DNA Synthesizer, Model 394, using phosphoamidite chemistry.

EXAMPLE 2

Design and synthesis of degenerate 5' primer pools generic for mouse IgG light and heavy chains.

Degenerate primers were designed such that members of each set would hybridize to representatives of several gene families. This approach was undertaken because antibodies of future interest may belong to any of several gene families.

Sets of degenerate primers synthesized from the relatively conserved sequences of the heavy chain leader (V_H -leader), 5' end of the heavy chain gene variable region (V_H), or 5' end of the light chain gene variable region (V_L) were analyzed for amplification of the S107 family members from total RNA isolated from the PC-specific mouse hybridoma line R2-09. The 3'-end constant region of the light (C_L) and heavy chain (C_H1) genes have relatively little sequence variation. The most common type of antibody light chain is k and the predominant isotype for heavy chain from secondary cells is IgG. Thus, the 3' end primers used for the amplification of the kappa light chain and IgG1 heavy chain of anti-PC with T15-specific primers in Section V.C.1 were used in combination with the 5'-end degenerate primers.

The 5'-end heavy and light chain variable region degenerate primers were designed to contain a SalI restriction enzyme site at their 5' ends to aid in the cloning of the genes; however, no restriction enzyme sites are present in the degenerate V_H-leader primers.

Degenerate primers were designed using the data base of Kabat et al. (In: Sequences of Proteins of Immunological Interest, 4th Edition (1987) U.S. Dept. Health and Human Services), taking into account codon degeneracies for each amino acid in the conserved sequences of the leader and FR1 regions. The rationale for the design of this degenerate pool is described above in the Description section. The degenerate pool generic for the light chain is depicted in Figure 2 (92-099 to 92-102), for the heavy chain starting with the leader sequence in Figure 2 (92-107 to 92-108), and for the heavy chain starting with coding region for the mature protein in Figure 2 (92-095 to 92-098 and 92-109 to 92-110).

Each of the degenerate sequences was synthesized in a separate sub-pool. The following description of the synthesis of the degenerate pool for the light chain [Figure 2, sequences 92-099, 92-100, 92-101, and 92-102] illustrates the principle for synthesis of degenerate pools. Briefly, the oligonucleotide synthesis of sub-pool 92-099 was begun by the addition starting at the 3' end of A, then C, followed by C. Then A was specifically added at the degenerate site four nucleotides from the 3' end. Oligonucleotide synthesis was continued with the addition of C followed by T. Then A and G were added to the reaction mix in 1:1 proportion for addition at the heterogeneous site 7 nucleotides from the 3' end. Synthesis was continued with the specific addition of A followed by C. Then G, C, and A were added

to the reaction mix in the proportion of 1:1:1 for addition at the heterogeneous site 10 nucleotides from the end. Oligonucleotide synthesis was continued in this manner until degenerate sub-pool 92-099 was complete.

Each degenerate sub-pool was synthesized in this manner, resulting in four separate primer sub-pools. Then the sub-pools were combined in equal proportion to form the light chain 5' degenerate primer pool generic for mouse light chain immunoglobulin. By this method, it was assured that the total pool would have an equal representation of A, T, C, and G at the position four nucleotides from the 3' end.

EXAMPLE 3

3SR Amplification of RNA Encoding Anti-PC Monoclonal Antibody, and Synthesis of Anti-PC Single Chain Antibody.

This experiment demonstrated that the degenerate primer pool described in Example 2 yields the same results in 3SR as primers specific for the known antibody sequence (Example 1). Moreover, an anti-PC single chain antibody was produced using the 3SR amplification products.

Preparation of RNA from hybridoma extracts:

Total RNA from two PC-specific hybridomas, R2-26 and R2-09, was extracted by an acid guanidinium isothiocyanate/phenol-chloroform extraction protocol (Chomczynski and Sacchi, Anal. Biochem., 162:156-159, 1987). Purification of poly(A)⁺ RNA from total RNA isolated from R2-26 was performed with the Dynal® Dynabeads® mRNA Purification Kit according to manufacturer instructions.

3SR amplification:

The basic technique of 3SR amplification is described in PNAS 87:1874-1878, 1990, Guatelli, et al. Briefly, RNA

was denatured in reaction mix containing primers at 65°C for 1 minute. Two separate 3SR amplifications for the heavy and light chain genes were performed, the first using the specific primers described in Example 1, and the second using the degenerate primer pool described in Example 2.

Annealing was conducted at 42°C for 1 minute, after which enzyme mix was added. Enzymes used were (1) AMV Reverse Transcriptase (Life Sciences) (2) T7 RNA polymerase (Bartels) and (3) E. coli RNaseH (Bartels). The mixture was incubated at 42°C for 1 hour, then frozen on dry ice and stored at -70°C.

Standard 3SR amplifications of 0.1 pmole of HIV-1 RNA with primers 88-211 and 88-347 for the envelope region were carried out with each amplification of antibody as a positive control for the amplification reaction. cDNA was generated with the original 3SR primers 92-(107,108), which hybridize to the 5' end of the heavy chain leader region. The concentration of each primer in the reaction was 0.375 μ M. Following the reverse transcription reaction, primer 92-004, which hybridizes to the 5' end of the heavy chain constant region (C_H1), was added to complete the primer pair for the PCR reaction. The concentration of primer 92-004 was 0.15 μ M.

The final concentration of each of the T15-specific primers in the 3SR, cDNA, and PCR reactions used in the successful isolation of heavy and light chain genes was 0.1 μ M, 1.0 μ M, and 0.2 μ M, respectively. The 3SR reaction was modified for the degenerate primers as follows: the concentration of each primer in the reaction was either 0.025 μ M or 0.25 μ M, and the reaction was allowed to proceed for 1.5 hours. The PCR reaction was modified by increasing the concentration of the 5'-

end degenerate primers for the cDNA reaction as described below.

Heavy chain, leader region: Aliquots of approximately 0.9 μ g of total RNA from PC-specific hybridoma line R2-09 were amplified by 3SR with primers 92-(107,108)/91-268* (Figure 2). Sequences for the degenerate primers 92-107 and 92-108 were obtained from Coloma et al. (BioTechniques, 11:152-156, 1991) and hybridize to the 5' end of the leader sequence of multiple heavy chain gene families. Specific primer 91-268* hybridizes to the 5' end of the heavy chain variable region (C_H1).

Heavy chain, variable region: Aliquots of approximately 0.9 μ g of total RNA from PC-specific hybridoma line R2-09 were amplified by 3SR with primers 92-(95-98)/91-268* (Figure 2). Sequences for degenerate primers 92-(95-98) hybridize to the 5' end of the variable region of multiple heavy chain gene families and contain a SalI restriction enzyme site at their 5' ends. Primer 91-268* hybridizes to the 5' end of the heavy chain constant region (C_H1).

Primers 92-109 and 92-110 hybridize to the heavy chain variable region.

Light chain variable region: Aliquots of approximately 0.9 μ g of total RNA from PC-specific hybridoma line R2-09 were amplified by 3SR with primers 92-(99-102)/92-002* (Figure 2). Sequences for the degenerate primers 92-(99-102) hybridize to the 5' end of the variable region of multiple light chain gene families and contain a SalI restriction enzyme site at their 5' ends. Primer 92-002* hybridizes to the 5' end of the light chain constant region (C_L).

Detection of 3SR products, slot blot analysis: 3SR

products were detected by immobilizing three serial dilutions of each reaction on a nylon filter (Bio-Rad Zeta-Probe®) utilizing a slot blot apparatus (Schleicher and Schuell). Aliquots corresponding to 2 μ l, 0.2 μ l and 0.02 μ l of the reaction were immobilized on the blot. Each blot included 3SR amplifications of HIV-1 as a positive control. The blots were probed with ^{32} P-labeled oligonucleotides specific for the RNA of interest. Duplicate filters were probed separately with oligonucleotides 92-008 and 92-009 (Figure 2), which are anti-sense and sense probes, respectively, homologous for a relatively conserved sequence in the J-C_H1 region, specifically J_H1, J_H2, and J_H4. Primers 92-012 and 92-013 hybridize to the 5' end of the constant region of the light chain.

Northern analyses: Northern blot analyses were performed on a NuPAGE 8% RNA Gel Kit (Novex) according to the manufacturer's instructions. Aliquots of 3SR amplification products were denatured by diluting each sample 1:1 in 2xNuPAGE Urea Sample Buffer (Novex) and heating for 2 min at 85°C before separation. Following electrophoresis, transfer of the nucleic acids onto a nylon filter (Bio-Rad Zeta-Probe®) was carried out for 45 min at 0.4 amps in 1xNuPAGE Running Buffer (Novex) on a transfer apparatus (Hoefer, TE22). The DNA was cross-linked to the filter by UV irradiation at 0.125 joules/cm. Each blot included a 3SR amplification of HIV-1 as a positive control. The blots were probed with ^{32} P-labeled oligonucleotides specific for the gene of interest. Either a MspI digest of pBR322 DNA (New England Biolabs) that had been ^{32} P-labeled or a 0.16-1.77 Kb RNA Ladder (Gibco BRL) was included on the blot as molecular weight markers. The filter was probed with oligonucleotides 92-009 and 92-013, which hybridize to the 3' end of the heavy chain variable region (J_H1, J_H2,

J_H4-C_H1) and the 5' end of the light chain constant region (C_L), respectively.

Further amplification by PCR: For the T-15 specific primers, aliquots of 2 μ l of 3SR amplification reactions of hybridoma RNA extracts were further amplified by RT/PCR essentially according to the manufacturer's specifications (Perkin Elmer Cetus GeneAmp® RNA PCR Kit).

The thermal cycle parameters with T-15 specific primers were: 1 cycle for 2 min at 94°C; 3 cycles of 1 min at 94°C for denaturation, 1.5 min at 42°C for primer annealing, and 2 min at 72°C for elongation; 30 cycles of 1 min at 94°C, 1 min at 64°C, and 2 min at 72°C; followed by 10 min at 72°C and a 4°C soak. The thermal cycle parameters for the degenerate primers were: 2 cycles of 1 min at 94°C for denaturation, 1 min at 42°C for primer annealing, and 2 min at 72°C for elongation; 30 cycles of 1 min at 94°C, 1 min 60°C, and 2 min at 72°C; followed by 10 min at 72°C and a 4° soak.

Southern analyses: Southern blot analyses were performed on 5- μ l aliquots of PCR amplification products separated on pre-cast 6% TBE polyacrylamide gels (Novex) as described (Engelhorn and Raab, BioTechniques, 11:594-596, 1991). Following electrophoresis, the gel was soaked for 10 min in 0.05 M NaOH and 5 min in 1xTBE. Transfer of the nucleic acids onto a nylon filter (Bio-Rad Zeta-Probe®) was carried out for 45 min at 0.4 amps in 1xTBE buffer on a transfer apparatus (Hoefer, TE22). After the transfer, the nylon membrane was washed for 5 min each in 0.1 M NaOH and H₂O. The DNA was cross-linked to the filter by UV irradiation at 0.15 joules/cm². The blots were probed with ³²P-labeled oligonucleotides specific for the gene of interest. The blots included an MspI digest of pBR322 DNA (New England Biolabs) that had been ³²P-labeled as molecular weight markers.

Results:

T15-specific primers. In slot blot and Northern analyses of the 3SR amplification products, heavy chain variable region products were detected in amplifications of R2-09 total RNA, and light chain variable region products were detected in amplifications from R2-26 poly(A)+ RNA. Northern analyses of 3SR amplifications and agarose gel analysis of PCR re-amplifications indicated that heavy and light chain variable region products generated from T15-specific primers and R2-09 total RNA and/or R2-26 poly(A+) RNA were of the expected sizes.

Degenerate primers: The degenerate primer pools generic for mouse IgG families yielded similar results in 3SR amplification as the the T15-specific primers. Thus these degenerate primer pools are suitable for the isolation of heavy and light chain variable region IgG genes of unknown gene types from secondary mouse spleen fragments producing monoclonal antibodies of desired affinity and specificity for a target antigen.

Synthesis of anti-PC single chain antibody: The 3SR amplification products of anti-PC light and heavy chains from hybridoma R2-09 were cloned into pBR322 as described below for anti-E2 antibodies. Plasmids pSHC-5 and pSLC-2 contained heavy and light chain anti-PC clones, respectively (Figure 4). pSHC-5 and pSLC-2 were linearized with PstI, and the heavy and light chain inserts were joined using a two-step PCR reaction. The first PCR reaction employed primer pairs 92-194 and 92-188, and 92-195 and 92-201 (Table 1) to amplify the heavy and light chain inserts, respectively (Figure 4). Primers 92-188 and 92-201 contain overlapping linker sequences based on the Genex 212 linker (Bird, R.E., et al., 1988 Science 242:423-426). The PCR fragments produced by the first PCR step were then joined by a second PCR reaction with the primer pair 92-194 and 92-

195 (Figure 4, Table 1). The resulting PCR fragment contained SalI and XmaI cloning sites at the termini (figure 4).

Table 1

Oligonucleotides used for the Synthesis of Anti-PC Single Chain Antibody

Primer	Sequence	Strand ¹	Target Gene ²	Restriction Sites ³
92-188	AGAGCTCTTACCACTACCGGAAGTAGATGAGGAGACGGTGACCGTGGTCCCTGC	AS	V _H	none
92-194	ACTAGTCGACGAGGTGAAGCTGGTGAATCTGGAGGA	S	V _H	<i>Sal</i> I
92-195	ACTACCCGGGCCGTTTCAGCTCCAGCTGGTCCACGA	AS	V _L	<i>Xma</i> I
92-201	GGTAGTGGTAAGAGCTCTGAAGGTAAAGGTATTGTGATGACTCAGTCTCCAACCTT	S	V _L	none

¹S (sense) refers to sequences that are identical to the target RNA, whereas AS (anti-sense) refers to the sequences that are complementary to the target RNA.

²Heavy chain variable region (V_H), light chain variable region (V_L).

³*Sal*I and *Sma*I sites are underlined in sequence.

Construction of pT15-110 expression vector: Plasmid pT15-110 was constructed for the expression of anti-PC sc antibody in E. coli. In vector pT15-100, the gene for PC sc antibody is under the regulation of the alkaline phosphatase (phoA) promoter region and the Bacillus thuringiensis cry transcription terminator (Wong, H.C., et al., 1986 PNAS 83:3233-3237). The phoA leader sequence is used to direct secretion of the antibody. Additionally, in pT15-100, a nucleotide sequence encoding five histidine residues is fused to the 3' end of the PC sc antibody gene. These histidine residues function as a metal binding site (MBS) and can be used in a rapid partial purification of the antibody. A nucleotide sequence encoding the 13 amino acids from the carboxy terminus of the human c-myc protein (Ramsey, G., et al., 1985 Mol. Cell Biol. 5:3610-3616) is also fused to the 5' end of the antibody gene. These amino acids provide an immunological tag useful for monitoring the expression and purification in Western blots and functionality in ELISA assays.

Expression vector P15-110 was constructed as follows (Figure 4). The phoA promoter and leader sequence and the cry terminator were obtained in plasmid pSYC 1087. Oligonucleotide 92-183 (5'GGCGCCGTCGCCCCGGGCATCACCATCATCACTAGGGATCC 3') was inserted into the narI/BamHI sites (*italics*) of pSYC 1087 to form vector pMBS4. This resulted in the insertion of the codons for five histidine residues as well as restriction enzyme sites NarISaI, and SmaI for subsequent cloning steps.

The original polylinker positioned 5' to the phoA promoter in pSYC 1087 was removed by digestion of pMBS-4 with ClaI and XbaI. The ends were repaired by Klenow DNA

polymerase and religated to form plasmid pMBS-101.

To form pPHO 101, oligonucleotide 93-011
(5'GAACAAAACTCATCTCAGAAGAGGATCTGGGTGCAGTCGAC 3') was
5 inserted into pMBS 101 which had been digested with NarI,
treated with Klenow DNAPolymerase and digested with SalI.
This resulted in the addition of the sequence encoding
the c-myc tag.

10 The anti-PC sc antibody gene, constructed by a series of
PCR reactions described above (Figure 4) was then
inserted into the SalI/XmaI sites of pPHO 101 to form
expression vector pT15-110.

15 Expression of anti-PC sc antibody: Cell culture
conditions.

E. coli strain MM294 was transformed with pT15-110 and
used as a host for the expression of anti-PC sc antibody.
Cells transformed with plasmid pPHO-101 served as a
20 negative control. Individual E. coli transformants were
grown at 30°C to approximately 4-5 OD₆₀₀ in phosphate
medium (1x MOPS, 0.4% glucose, 0.15% vitamin-free
casamino acids, 10 mg/ml B1, 100 mg/ml ampicillin)
(Neidhart, F.C., et al., 1974 Enterobacteria. J.
25 Bacteriol. 119:736-747) containing 10 mM KH₂PO₄. This
media represses phoA expression. The cells were washed
with phosphate medium and then suspended in phosphate
medium containing 0.1 mM KH₂PO₄ at an OD₆₀₀ of
approximately 0.08. E. coli cultures were grown under
30 low phosphate conditions for 7 hours to achieve maximal
anti-PC sc antibody expression.

Cell lysis and antibody purification: Cell lysis was
accomplished by resuspending transformed cells (20 OD₆₀₀)
35 in 1.0 ml of sonication buffer (50 mM Na phosphate, pH
8.0, 300 mM NaCl, 0.25% Tween-20, 0.1 mM EGTA, 1 mM

phenylmethylsulfonylchloride). The cells were frozen on dry ice/ethanol, thawed and sonicated on ice (10 cycles of 10 second bursts with 1 min cooling at 20 watts with a Bronson sonifier, [Danbury, CT], Model 450). The lysed cells were centrifuged at 11,000 rpm for 20 min at 4°C. The supernatant represented the total soluble protein. The total protein concentration in the supernatant was measured by the Lowry method with a DC Protein Asssay Kit (BioRad, Richmond, CA).

Partial purification of anti-PC sc antibody: Anti-PC sc antibody was partially purified from cell lysates using a Nickel (Ni)-NTA resin (Qiagen, Chatsworth, CA) according to the manufacturer's recommendations. Briefly, a 50% slurry of Ni-NTA resin (previously equilibrated in sonication buffer) was added to an aliquot of supernatant cell lysate and agitated for 1 hour at 4°C. The resin was centrifuged at 14,000 rpm and the unbound fraction was collected. The resin was then washed with 1.0 ml aliquots of 10 to 100 mM imidazole dissolved in sonication buffer was applied to the resin in 1.0 ml aliquots. Aliquots were analyzed by Western blot using monoclonal antisera specific to c-myc (Oncogene Sciences, Uniondale, NY). ELISA assays were performed by coating each well of a 96 well microtiter plate with 50 μ l of 50 μ g/ml of phosphorylcholine conjugated to bovine serum albumin (PC-BSA) in 0.05 M carbonate buffer. Aliquots of E. coli lysates or fractions eluted from the Ni-NTA resin were added and reacted at room temperature for 4 hours. The secondary antibody reaction, in which the mouse anti-c-myc binds to the anti-PC sc antibody, was allowed to incubate 4 hours at room temperature. The bound anti-c-myc antibody was visualized by incubation with alkaline phosphatase-labeled goat anti-mouse Ig antibody followed by reaction with p-nitrophenyl-phosphate (NPP). After color development, the plates were read at 405 nm on a

Dynatech (Chantilly, VA) MR5000 microtiter plate reader.

Results: Processed (approx. 32 kDa) and unprocessed (approx. 33 kDa) monomeric as well as dimeric (approx. 60 kDa) and trimeric (approx. 90 kDa) forms of sc anti-PC antibody were observed on Western blot analyses of whole cells lysates. However, Western blot analysis of a periplasmic space fraction extracted with Tween 20 and EGTA revealed that approximately 75% of the total sc antibody expressed by E. coli is processed and transported into the periplasmic space where it apparently is associated with the inner membranes requiring release by non-ionic detergent.

Partial purification of the anti-PC sc antibody was achieved by adsorption through the metal binding polyhistidine tract inserted at the 3' end of the sc antibody. Denaturation conditions of 6M urea was required for the sc antibody to bind quantitatively to the resin, otherwise the majority of the sc antibody was observed in the unbound fraction. The peak of the elution profile of sc antibody occurred at 40 mM imidazole.

In the quantitative ELISA assay, the fraction eluted with 40 mM imidazole from the Ni-NTA resin was the only material that demonstrated quantitative binding to the immobilized phosphorylcholine. Because the undenatured anti-PC sc antibody that was present in the unbound fraction from the Ni-NTA resin did not react in the ELISA assay, it may be concluded that the majority of the anti-PC sc antibody made in E. coli is folded such that it does not recognize the immobilized phosphorylcholine and/or the c-myc tag is also unavailable for the second antibody. Such improperly folded sc antibody can be denatured and refolded into a functional form as

previously demonstrated for an anti-PC sc antibody (Glockshuber, R., et al., 1992 Biochem. 31:1270-1279). The amount of the sc anti-PC antibody produced by pT15-110 in E. coli was estimated using silver-stained SDS-PAGE to be approximately 0.1% of the total cell protein.

EXAMPLE 4

ISOLATION OF ANTI-E2 HEAVY AND LIGHT CHAIN GENES FROM RNA ISOLATED FROM SECONDARY MOUSE SPLEEN FRAGMENTS

Donor BALB/c mice were each injected twice with 100 μ g estradiol b coupled to Limulus polyphemus hemocyanin (Hy) (gift from F. Boches) at two month intervals, as described in Linton, et al(supra). One to two months after the second injection, approximately $1-2 \times 10^8$ whole spleen cells were collected from the donor mouse. MHC syngeneic, Hy (carrier) primed recipient mice were lethally irradiated with 1300R, and approximately 4×10^6 donor spleen cells were transferred intravenously. Within 24 hours of cell transfer, the recipient's spleen was removed and dissected into 1-mm cubic fragments. Each spleen fragment was cultured in individual wells of a microtiter dish in the presence of E2-Hy for 2-3 days. Five to seven days later, culture fluids from the wells were screened by an ELISA assay for antibodies specific for E2 (anti-E2). When limiting numbers of B cells are transferred, subsequent responses are expected to be monoclonal. In this experiment, most of the microtiter wells were positive, suggesting that responders were likely to be polyclonal. Antibodies obtained from positive fragments were further analyzed for relative affinity by an inhibition ELISA assay wherein 10^{-5} to 10^{-8} molar dilutions of competing E2 were added to the culture-produced antibodies during the ELISA.

Culture fragments which were positive for anti-E2 antibody were subjected to extraction of total nucleic acids (Stallcup, M.R., et al., (1983) J Biol Chem 258:2802-2807). This total nucleic acid was used for 3SR amplification and subsequent cloning of Ig light and heavy chain cDNAs.

An aliquot of total RNA from each E2-specific secondary spleen fragment was amplified by 3SR as described in Example 3 with the following exceptions: the concentration of each primer in the reaction was 0.25 μ M, and the reaction was allowed to proceed for 90 minutes to 1.5 h. Two pools of generic heavy chain primers were used to amplify the anti-E2 mRNAs: 92-(107,108) (leader) or 92-109/92/110 (variable) (Figure 2). The 3'-end primer used was 91-267, which hybridizes to the C_{H1} region of the heavy chain mRNA. Generic light chain primers 92-99 to 92-102 (Figure 2) hybridize to the 5'-end of the light chain variable region and are composed of four pool of degenerate oligonucleotides. Primer 92-002 hybridizes to the C_L region.

To convert the 3SR RNA amplification products to DNA, 2 μ l of each 50 μ l 3SR amplification reaction was amplified by the reverse transcriptase (RT)/polymerase chain reaction (PCR) protocol recommended by the manufacturer [Perkin Elmer Cetus, (GENEamp®RNA kit)]. Heavy chain oligonucleotide primers 92-107/92/108 (leader region) and 92-109/92-110 (variable region) (Figure 2) were used with AMV RT to synthesize the first strand of cDNA. Following the RT reaction, primer 92-140 (5'ACTAGAATTTCAGTGGATAGACAGATGGGGGTG 3') which contains an inserted EcoR1 site was used to complete the PCR primer pairs. For the light chain PCR reaction, a primer pool consisting of 92-99 to 92-102 (Figure 2) was used to create the first strand cDNA. Primer 92-012 (Figure 2)

was then used to complete the PCR primer pairs. During the RT reaction, the primer concentration was set at 1.25 mM, whereas, during the PCR reaction, the final concentration of each 5'- and 3'-end primer was 0.25 and 1.0 mM, respectively. The conditions used for the thermal cycling were: 30 cycles, each cycle consisting of 1 minute at 94° (denaturation), 1 min at 55° (annealing), 2 minutes at 72° (primer extension), followed by 10 minutes at 72° and a 4° termination step.

Northern blot analysis was performed on the 3SR amplification products with a NuPage 8% RNA Gel Kit (Novex, San Diego, CA) according to the manufacturer's instructions. Aliquots (10 μ l) of 3SR amplification products were denatured by diluting each sample with an equal volume of 2x NuPage Urea Sample Buffer followed by heating for 2 minutes at 85°C before electrophoresis. Following electrophoresis, transfer of the nucleic acids onto a Zeta Probe® nylon filter (BioRad, Richmond, CA) was carried out for 45 minutes at 0.4 amps in 1x NuPage Running Buffer on a TE22 transfer apparatus (Hoefer Scientific, San Francisco, CA). The DNA was cross-linked to the filter by UV irradiation at 0.15 joules/cm². The filters were probed with ³²P-labeled oligonucleotides 92-009 or 92-013 (Figure 2). An MspI digest of pBR322 DNA (New England Biolabs, Beverly, MA) which had been pre-labeled with ³²P was included on each blot as molecular weight markers.

Southern blot analyses were performed on aliquots (1-5 μ l) of PCR amplification products separated on pre-cast 6% TBE polyacrylamide gels (Novex, San Diego, CA). Following electrophoresis, the gel was soaked for 10 minutes in 0.05 M NaOH and 5 minutes in 1x TBE. Transfer of the nucleic acids onto Zeta Probe® nylon filters was carried out for 45 minutes at 0.4 amps in 1x TBE buffer

on a TE22 transfer apparatus. After the transfer, the nylon membrane was washed for 5 minutes each in 0.1 M NaOH and H₂O. The DNA was cross-linked to the filter by UV irradiation at 0.15 joules/cm². The filters were probed with ³²P-labeled oligonucleotides 92-009 or 92-013 (Figure 2).

Cloning, nucleic acid sequencing, and computer analysis of anti-E2 heavy and light chain clones.

Because generic primers were used to amplify heavy and light chain immunoglobulin mRNAs, the amplification products were cloned and the clones were individually analyzed by sequencing. A 10 µl aliquot of the anti-E2 leader heavy chain amplification reaction was treated with Klenow DNA polymerase and then digested with EcoRI. The blunt end/EcoRI-cleaved fragments were gel purified and ligated into pUC18 which has been previously digested with SmaI and EcoRI and treated with calf alkaline phosphatase (CAP). The anti-E2 heavy chain variable region amplification reaction (10 µl) was digested with SalI and EcoRI because both restriction endonuclease sites were engineered into primers 92-(109-110) and 92-140, respectively, for cloning purposes. The digested PCR fragments were gel purified and ligated into pUC18 which had been previously digested with SalI and EcoRI and treated with CAP. The anti-E2 light chain amplification reaction (10 µl) was treated with Klenow DNA polymerase and digested with SalI. The PCR fragments were gel purified and ligated into SmaI and SalI digested pUC18 or pBR322 which had been treated with CAP. Dilutions of each ligation were used to transform competent Escherichia coli MC1061 (recA⁺ or HB101 (recA⁻ cells).

Nucleotide sequence analyses of pUC18 and pBR322 heavy and light chain clones were performed by the modified

dideoxy nucleotide chain termination method described by Hsiao, et al (Nucleic Acid Res. 19:2787). Computer analysis of the derived sequence was performed with MacVector (IBI, New Haven, CT) and Line-Up from UWGCG.

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Cloning and Sequence Analysis: During the initial screening step, cells in a splenic fragment, D₁A₆, were identified as producing antibody specific for estradiol with an affinity constant of approximately 10⁷ liters per mole. Total nucleic acid from cells of this fragment was amplified by 3SR with the light and heavy chain generic primers shown in Figure 2. The 3SR RNA products of the heavy and light chain amplifications were converted to DNA, digested with the appropriate restriction endonucleases and cloned into either pUC 18 or 19.

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A total of 39 heavy and 36 light chain productive clones derived from fragment D₁A₆ were sequenced. Comparative sequence analysis of the clones using the UWGCG LineUP program revealed the presence of four alleles each for the heavy and light chain groups (Table 2). The nucleotide sequence representative of clones from each allelic group is presented in Figure 5. The heavy chain clones from groups 2, 3 and 4 exhibited 81-86% similarity with the J558 V_H heavy chain family (Kabat, et al., supra; Brodeur, P.H., et al., J Exp Med 168:2261-2278), whereas clones from group 1 were marginally similar to the S107 (79.7%) V_H family. However, comparison of the sequences of clones of group 1 to sequences in GENBANK revealed a 96% similarity to a mouse anti-DNA rearranged heavy chain variable region (Kofler, R., et al., J Clin Invest 82:852-860). Only in clones from groups 2 and 4 could the family of the D_H minigenes be unambiguously determined as being from DFL16.1 and DST4 minigenes, respectively (Kabat, et al., supra). The J_H minigenes utilized were J_H 2, 3 and 4. The light chain clones from fragment D₁A₆ also

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consisted of four V_k alleles (Figure 5B). Clones from groups 2 and 4 were highly similar (96-97%) to the 19/28 V_k family (Strohol, R., et al., Immunogenetics 30:475-493), whereas clones from groups 1 and 3 were similar to the $V_{k4/5}$ (92%) and $V_k 10$ (88%) families, respectively. Light chain clones used the J_{k5} minigene while clones from group 1 used the J_{k4} minigene.

Unlike the variable region degenerate primers, the leader region primers allowed for unambiguous determination of the sequences present at the amino terminus of the V_H minigene. In group 1 clones, derived from the leader region primers, the nucleotide sequences predicted amino acids Val-5, Glu-6 and Thr-7 in place of Gln-5, Gln-6, and Ser-7 as predicted by using the generic variable region primers. Likewise, in

TABLE 2

Heavy and Light Chain Clones Obtained from Spleen Fragment Culture D₁A₆

Alleles	# of Clones Analyzed	Minigene Families		
		V (% homology)	D	J
V _H Clones				
1	11	S107 (79)	DSP 2,3, 4, 6	J _H ²
2	6	J558 (81)	DFL 16.1	J _H ³
3	15	J558 (86)	DSP 2,3, 7	J _H ⁴
4	7	J558 (83)	DST 4	J _H ³
V _L Clones				
1	16	V _K 4/5 (92)	-	J _K ⁴
2	10	V _K 19/28 (96)	-	J _K ⁵
3	6	V _K 10 (88)	-	J _K ⁵
4	3	V _K 19/28 (97)	-	J _K ⁵

group 4, clones derived from the leader primers contained the codon for the amino acid Leu at position 3 in place of Lys as predicted by the variable region primers.

5 The clones sequenced in each of the four groups of light and heavy chain variable regions were productive, indicating that more than one memory B cell colonized the splenic fragment D₁A₆. To avoid obtaining multiple sequences, limiting dilution of transferred lymphocytes
10 can be performed to obtain only one colonizing antigen responsive B cell. However, even in this case with multiple heavy and light chain alleles represented, it is relatively straightforward to join together the four
15 light and heavy chain clones into all 16 possible anti-E2 antibody combinations. One of the combinations will reflect the original V_H and V_L pairings. Even in this case, the need to create and screen larger phage display libraries is avoided.

EXAMPLE 5

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Production of a recombinant monoclonal antibody against estradiol.

RNA encoding heavy and light chains of anti-estradiol, as produced in Example 4, is converted to cDNA. The cDNA,
25 when converted to double stranded DNA, is incorporated into expression vectors, which are then transfected into E. coli host cells. The host cells are induced to express the heavy chain and the light chain recombinant proteins for the anti-estradiol antibody. The heavy and
30 light chains associate and form the appropriate disulfide bridges to form the complete anti-estradiol antibody. The antibody is isolated and purified from the host cell debris. The resulting recombinant monoclonal antibody possesses the desired specificity and affinity
35 for estradiol as identified in the assays of the positive spleen fragment culture in Example 4.

We claim:

1. A method for obtaining RNA encoding a predetermined chain of a desired antibody against a specific antigen, comprising:

- 5 a) isolating a B cell population from a first mammal, said B cell population containing responsive B cells,
- b) injecting an amount of said B cell population into a second mammal, said second mammal having no viable
10 endogenous B cells,
- c) maintaining said second mammal for sufficient time to allow said injected B cells to colonize the spleen of said second mammal,
- d) removing the spleen of said second mammal,
- 15 e) cutting said spleen into fragments of a predetermined size, said size being sufficiently small so that essentially each fragment contains no more than one responsive B cell,
- f) maintaining each fragment in a separate
20 culture, each culture containing a culture medium,
- g) contacting each fragment with said specific antigen,
- h) maintaining each fragment in culture for sufficient time to allow proliferation of responsive B
25 cells within each fragment and antibody production by at least some of the responsive B cells,
- i) assaying the culture medium of each well for content of desired antibody, thereby identifying a positive fragment containing antibody-producing B cells,
- 30 j) extracting heterogeneous RNA from said positive fragment, said heterogeneous RNA containing a first target RNA and a second target RNA, said first target RNA encoding the light chain of said desired antibody, and said second target RNA encoding the heavy chain of said
35 desired antibody,
- k) contacting said heterogeneous RNA with a first

degenerate primer pool or with a second degenerate primer pool, said first degenerate primer pool containing primer nucleotide sequences complementary to at least a portion of said first target RNA, said second degenerate primer pool containing primer nucleotide sequences to at least a portion of said second target RNA, said first or second primer nucleotide sequences hybridizing to said first or second target RNA respectively,

1) amplifying said first target RNA or said second target RNA using isothermal self-sustained sequence replication, thereby obtaining an amplified RNA consisting essentially of said first target RNA or said second target RNA.

2. The method of claim 1 wherein step (k) comprises contacting said heterogeneous RNA with said first degenerate primer pool, said first primer nucleotide sequences hybridizing with said first target RNA, and step (l) comprises amplifying said first target RNA using isothermal self-sustained sequence replication, thereby obtaining an amplified RNA consisting essentially of said first target RNA encoding the light chain of said desired antibody.

3. The method of claim 1 wherein step (k) comprises contacting said heterogeneous RNA with said second degenerate primer pool, said second primer nucleotide sequences hybridizing with said second target RNA, and step (l) comprises amplifying said second target RNA using isothermal self-sustained sequence replication, thereby obtaining an amplified RNA consisting essentially of said second target RNA encoding the heavy chain of said desired antibody.

4. The method of claim 1 wherein, prior to step (a), said first mammal is injected with said antigen.

5. The method of claim 1 further comprising injecting said second mammal with at least a portion of said antigen, thereby stimulating T helper cell function in said second mammal.

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6. The method of claim 1 wherein said first and said second mammals are congenic mice.

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7. The method of claim 1 wherein said first and said second mammals are members of different species.

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8. The method of claim 6 wherein said amount of injected B cells is sufficiently low such that the second mammal's spleen will be on the average colonized by not more than one responsive B cell in each 1mm^3 segment of spleen tissue.

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9. The method of claim 8 wherein said fragment size is approximately 1mm^3 .

10. The method of claim 8 wherein steps (g) through (i) are repeated at least once.

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11. The method of claim 2 wherein said first mammal is a mouse, said first target RNA encodes a mouse immunoglobulin light chain, and said generic degenerate primer pool comprises a 5' primer pool having an equal proportion of I, II, III, and IV, wherein

I is:

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GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCA CCA

II is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCT CCA

III is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCC CCA

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IV is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCG CCA

wherein $X_1 = T$ or C , $X_2 = T$ or C , $X_3 = G$ or C , $X_4 = G$, C , or T , $X_5 = A$, C , or G , and $X_6 = A$ or G .

5 12. The method of claim 11 wherein said immunoglobulin is an IgG, and said generic degenerate primer pool further comprises a 3' primer specific for mouse IgG light chain constant region, said 3' primer having the sequence designated 92-002* in Figure 2, said degenerate primer pool comprising one parts of said 3' primer and
10 four parts of said 5' primer pool.

13. The method of claim 11 wherein said immunoglobulin is an IgG, and said generic degenerate primer pool further comprises a 3' primer specific for mouse IgG
15 light chain constant region, said 3' primer having the sequence designated 92-006 in Figure 2, said degenerate primer pool comprising one part of said 3' primer and four parts of said 5' primer pool.

20 14. The method of claim 3 wherein said first mammal is a mouse, said second target RNA encodes a mouse immunoglobulin heavy chain and said generic degenerate primer pool comprises a 5' primer pool having an equal proportion of I and II, wherein

25 I is:

ATG X_1X_2 TTX₃ X_4GG X_5TX_6 AX₇C TX₈G X_9TT

II is:

ATG $X_{10}AA$ TGX₁₁ AX₁₂C TGG GTX₁₃ $X_{14}TX_{15}$ CTC T

30 wherein $X_1 = G$ or A , $X_2 = G$ or C , $X_3 = G$ or C , $X_4 = T$ or G , $X_5 = T$ or C , $X_6 = A$ or C , $X_7 = A$ or G , $X_8 = G$ or T , $X_9 = G$ or A , $X_{10} = G$ or A , $X_{11} = G$ or C , $X_{12} = G$ or C , $X_{13} = C$ or T , $X_{14} = T$ or A , $X_{15} = T$ or C .

35 15. The method of claim 14 wherein said immunoglobulin is an IgG, and said generic degenerate primer pool

further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 91-267* in Figure 2, said degenerate primer pool comprising one part of said 3' primer and two parts of said 5' primer pool.

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16. The method of claim 14 wherein said immunoglobulin is an IgG, and said generic degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 91-268* in Figure 2, said degenerate primer pool comprising one part of said 3' primer and two parts of said 5' primer pool.

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17. The method of claim 14 wherein said immunoglobulin is an IgG, and said generic degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 92-004 in Figure 2, said degenerate primer pool comprising one part of said 3' primer and two parts of said 5' primer pool.

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18. The method of claim 3 wherein said first mammal is a mouse, said second target RNA encodes a mouse heavy chain and said generic degenerate primer pool comprises a 5' primer pool having an equal proportion of I, II, III, and IV, wherein

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I is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCA GG

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II is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCT GG

III is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCC GG

IV is:

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CAG GTX₁ CAA CTX₂ CAG CAX₃ TCG GG

wherein X₁ = G, C, or A, X₂ = G, C, or A, X₃ = A or G.

19. The method of claim 18 wherein said immunoglobulin is an IgG, and said generic degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 91-267* in Figure 2, said degenerate primer pool comprising one part of said 3' primer and four parts of said 5' primer pool.

20. The method of claim 18 wherein said immunoglobulin is an IgG, and said generic degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 91-268* in Figure 2, said degenerate primer pool comprising one part of said 3' primer and four parts of said 5' primer pool.

21. The method of claim 18 wherein said immunoglobulin is an IgG, and said generic degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 92-004 in Figure 2, said degenerate primer pool comprising one part of said 3' primer and four parts of said 5' primer pool.

22. A method for producing a recombinant protein having a desired binding affinity and specificity for a specific antigen, comprising:

- a) isolating a B cell population from a first mammal, said B cell population containing responsive B cells,
- b) injecting an amount of said B cell population into a second mammal, said second mammal having essentially all endogenous B cells destroyed,
- c) maintaining said second mammal for sufficient time to allow said injected B cells to colonize the spleen of said second mammal,

d) removing the spleen of said second mammal,
e) cutting said spleen into fragments of a predetermined size, said size being sufficiently small so that essentially each fragment contains no more than one responsive B cell,

f) maintaining each fragment in a separate culture, each culture containing a culture medium,

g) contacting each fragment with said specific antigen,

h) maintaining each fragment in culture for sufficient time to allow proliferation of responsive B cells within each fragment and antibody production by at least some of the responsive B cells,

i) assaying the culture medium of each well for content of desired antibody, thereby identifying a positive fragment containing antibody-producing B cells,

j) extracting heterogeneous RNA from said positive fragment, said heterogeneous RNA containing a first target RNA and a second target RNA, said first target RNA encoding the light chain of said desired antibody, and said second target RNA encoding the heavy chain of said desired antibody,

k) contacting said heterogeneous RNA with a first degenerate primer pool, said first primer pool containing nucleotide sequences complementary to at least a portion of said first target RNA,

l) amplifying said first target RNA using isothermal self-sustained sequence replication, thereby obtaining an amplified RNA consisting essentially of said first target RNA,

m) synthesizing a first cDNA corresponding to said first target RNA,

n) contacting said heterogeneous RNA from step (j) with a second degenerate primer pool, said second primer pool containing nucleotide sequences complementary to at least a portion of said second target RNA,

- o) synthesizing a second cDNA corresponding to said second target RNA,
- p) constructing at least one expression vector containing at least a portion of said first cDNA and at least a portion of said second cDNA,
- q) delivering said vector into host cells,
- r) causing said host cells to express at least one recombinant protein encoded by said vector, and
- s) isolating said recombinant protein.

23. The method of claim 22 wherein, prior to step (a), said first mammal is injected with said antigen.

24. The method of claim 22 further comprising injecting said second mammal with at least a portion of said antigen, thereby stimulating T helper cell function in said second mammal.

25. The method of claim 22 wherein said first and said second mammals are congenic mice.

26. The method of claim 22 wherein said first and said second mammals are members of different species.

27. The method of claim 25 wherein said amount of injected B cells is sufficiently low such that the second mammal's spleen will be on the average colonized by not more than one responsive B cell in each 1mm^3 segment of spleen tissue.

28. The method of claim 27 wherein said fragment size is approximately 1mm^3 .

29. The method of claim 22 wherein steps (g) through (i) are repeated at least once.

30. The method of claim 22 wherein said first mammal is a mouse, said first target RNA encodes a mouse immunoglobulin light chain, and said first degenerate primer pool comprises a 5' primer pool having an equal proportion of I, II, III, and IV, wherein

I is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCA CCA

II is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCT CCA

III is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCC CCA

IV is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCG CCA

wherein X₁=T or C, X₂=T or C, X₃=G or C, X₄=G, C, or T, X₅=A, C, or G, and X₆=A or G.

31. The method of claim 30 wherein said immunoglobulin is an IgG, and said first degenerate primer pool further comprises a 3' primer specific for mouse IgG light chain constant region, said 3' primer having the sequence designated 92-002* in Figure 2, said degenerate primer pool comprising one parts of said 3' primer and four parts of said 5' primer pool.

32. The method of claim 30 wherein said immunoglobulin is an IgG, and said first degenerate primer pool further comprises a 3' primer specific for mouse IgG light chain constant region, said 3' primer having the sequence designated 92-006 in Figure 2, said degenerate primer pool comprising one part of said 3' primer and four parts of said 5' primer pool.

33. The method of claim 22 wherein said first mammal is a mouse, said second target RNA encodes a mouse immunoglobulin heavy chain and said second degenerate

primer pool comprises a 5' primer pool having an equal proportion of I and II, wherein

I is:

ATG X₁AX₂ TTX₃ X₄GG X₅TX₆ AX₇C TX₈G X₉TT

II is:

ATG X₁₀AA TGX₁₁ AX₁₂C TGG GTX₁₃ X₁₄TX₁₅ CTC T

wherein X₁ = G or A, X₂ = G or C, X₃ = G or C, X₄ = T or G, X₅ = T or C, X₆ = A or C, X₇ = A or G, X₈ = G or T, X₉ = G or A, X₁₀ = G or A, X₁₁ = G or C, X₁₂ = G or C, X₁₃ = C or T, X₁₄ = T or A, X₁₅ = T or C.

34. The method of claim 33 wherein said immunoglobulin is an IgG, and said second degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 91-267* in Figure 2, said degenerate primer pool comprising one part of said 3' primer and two parts of said 5' primer pool.

35. The method of claim 33 wherein said immunoglobulin is an IgG, and said second degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 91-268* in Figure 2, said degenerate primer pool comprising one part of said 3' primer and two parts of said 5' primer pool.

36. The method of claim 33 wherein said immunoglobulin is an IgG, and said second degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 92-004 in Figure 2, said degenerate primer pool comprising one part of said 3' primer and two parts of said 5' primer pool.

37. The method of claim 22 wherein said first mammal is a mouse, said second target RNA encodes a mouse heavy chain and said second degenerate primer pool comprises a 5' primer pool having an equal proportion of I, II, III, and IV, wherein

I is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCA GG

II is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCT GG

III is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCC GG

IV is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCG GG

wherein X₁ = G, C, or A, X₂ = G, C, or A, X₃ = A or G.

38. The method of claim 37 wherein said immunoglobulin is an IgG, and said second degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 91-267* in Figure 2, said second degenerate primer pool comprising one part of said 3' primer and four parts of said 5' primer pool.

39. The method of claim 37 wherein said immunoglobulin is an IgG, and said second degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 91-268* in Figure 2, said second degenerate primer pool comprising one part of said 3' primer and four parts of said 5' primer pool.

40. The method of claim 37 wherein said immunoglobulin is an IgG, and said second degenerate primer pool further comprises a 3' primer specific for mouse IgG heavy chain constant region, said 3' primer having the sequence designated 92-004 in Figure 2, said second degenerate

primer pool comprising one part of said 3' primer and four parts of said 5' primer pool.

41. A method for making a total pool of generic degenerate 5' primers for the efficient amplification of RNA encoding a predetermined immunoglobulin chain from a population of heterogeneous RNA, comprising

a) identifying areas of sequence heterogeneity among all isotypes of said chain,

b) cataloging each nucleic acid substitution at each heterogeneous site,

c) selecting a 5' region comprising 10 to 30 nucleotides, said region encoding a leader sequence or a 5' coding region for said chain,

d) selecting from within said 5' region a first annealing sequence, said first annealing sequence comprising 6 to 9 nucleotides at the 3' end of said 5' region,

e) identifying each nucleotide substitution catalogued for each substituted site within said first annealing sequence,

f) synthesizing a separate pool of degenerate primers for each substitution at each substituted site within said first annealing sequence, such that each pool contains an equal representation of nucleic acid substitutions for that site, and

g) combining said separate pools into a total pool such that said total pool contains an equal representation of nucleotide substitutions at each substituted site within said first annealing sequence.

42. A pool of degenerate 5' primers, to be used in conjunction with a 3' primer, for the efficient amplification of RNA encoding a mouse immunoglobulin light chain from a population of heterogeneous RNA, said pool comprising an equal proportion of I, II, III, and

IV, wherein

I is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCA CCA

II is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCT CCA

III is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCC CCA

IV is:

GAX₁ ATX₂ GTX₃ CTX₄ ACX₅ CAX₆ TCG CCA

wherein X₁=T or C, X₂=T or C, X₃=G or C, X₄=G, C, or T, X₅=A, C, or G, and X₆=A or G.

43. A pool of degenerate 5' primers, to be used in conjunction with a 3' primer, for the efficient amplification of RNA encoding a mouse immunoglobulin heavy chain from a population of heterogeneous RNA, said pool having an equal proportion of I and II, wherein

I is:

ATG X₁AX₂ TTX₃ X₄GG X₅TX₆ AX₇C TX₈G X₉TT

II is:

ATG X₁₀AA TGX₁₁ AX₁₂C TGG GTX₁₃ X₁₄TX₁₅ CTC T

wherein X₁ = G or A, X₂ = G or C, X₃ = G or C, X₄ = T or G, X₅ = T or C, X₆ = A or C, X₇ = A or G, X₈ = G or T, X₉ = G or A, X₁₀ = G or A, X₁₁ = G or C, X₁₂ = G or C, X₁₃ = C or T, X₁₄ = T or A, X₁₅ = T or C.

44. A pool of degenerate 5' primers, to be used in conjunction with a 3' primer, for the efficient amplification of RNA encoding a mouse immunoglobulin heavy chain from a population of heterogeneous RNA, said pool having an equal proportion of I, II, III, and IV, wherein

I is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCA GG

II is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCT GG

III is:

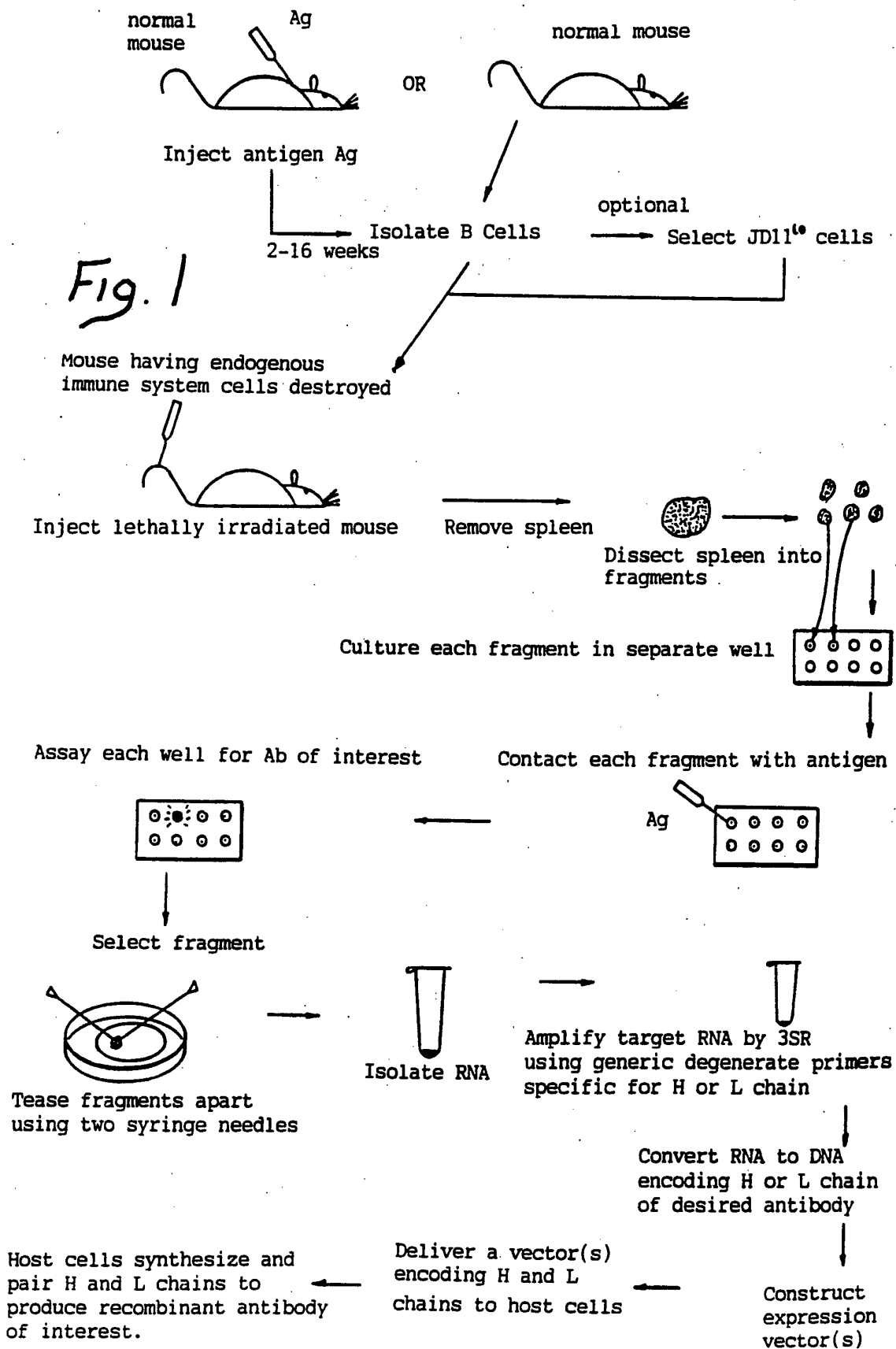
CAG GTX₁ CAA CTX₂ CAG CAX₃ TCC GG

IV is:

CAG GTX₁ CAA CTX₂ CAG CAX₃ TCG GG

wherein X₁ = G, C, or A, X₂ = G, C, or A, X₃ = A or G.

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OLIGONUCLEOTIDES FOR THE AMPLIFICATION AND DETECTION OF ANTIBODY FRAGMENTS

Primer ¹	Sequence	Use	Strand ²	Region	Enzyme Restriction Site
91-267	<u>AATTTAATACGACTCACTATAGGGAAGTGGATAGACAGATGGGGTG</u>	3SR/PCR	AS	C _H 1	none
91-268	<u>AATTTAATACGACTCACTATAGGGAAGTGGATAGTGGG CAGCAG</u>	3SR	AS	C _H 1	none
92-001	<u>AATTTAATACGACTCACTATAGGGAAGTGGATAGTGGG CAGCAG</u>	3SR	S	V _H	none
92-002	<u>AATTTAATACGACTCACTATAGGGAAGTGGATAGTGGG CAGCAG</u>	3SR	AS	C _L	none
92-003	<u>AATTTAATACGACTCACTATAGGGAAGTGGATAGTGGG CAGCAG</u>	3SR	S	V _L	none
92-004	<u>ATAGGGAATTCGATAGACAGATGGGGTG</u>	PCR	AS	C _H 1	EcoRI
92-005	<u>TCACTAAAGCTTGAGGTGAAGCTGGTGGAGTCTGGA</u>	PCR	S	V _H	HindIII
92-006	<u>ATAGGGAATTCGATAGACAGATGGGGTG</u>	PCR	AS	C _L	EcoRI
92-007	<u>TCACTAAAGCTTGACATTGTGATGACCCAGTCTC</u>	PCR	S	V _L	HindIII
92-008	<u>TCGTTTTGGCTGAGGAGACGGTGA</u>	Probe	AS	J1,J2,J4-C _H 1	none
92-009	<u>TCACCGTCTCCTCAGCCAAAACGA</u>	Probe	S	J1,J2,J4-C _H 1	none
92-010	<u>TCGTTTTGGCTGAGGAGACGGTGA</u>	Probe	AS	J3-C _H 1	none
92-011	<u>TCACTGTCTCTGCAGCCAAAACGA</u>	Probe	S	J3-C _H 1	none
92-012	<u>GGATACAGTTGGTGCAGCATCAGC</u>	Probe	AS	C _L	none
92-013	<u>GCTGATGCTGCACCAACTGTATCC</u>	Probe	S	C _L	none

Fig. 2

3 / 4

92-095	ACTAGTCGACCCAGGT(GCA)CAACT(GCA)CAGCA(AG)TCAGG	3SR/PCR	S	V _H	SalI
92-096	ACTAGTCGACCCAGGT(GCA)CAACT(GCA)CAGCA(AG)TCIGG	3SR/PCR	S	V _H	SalI
92-097	ACTAGTCGACCCAGGT(GCA)CAACT(GCA)CAGCA(AG)TCGGG	3SR/PCR	S	V _H	SalI
92-098	ACTAGTCGACCCAGGT(GCA)CAACT(GCA)CAGCA(AG)TCGGG	3SR/PCR	S	V _H	SalI
92-099	ACTAGTCGACCGA(TC)AT(TC)GT(GC)CT(GCT)AC(ACG)CA(AG)TCACCA	3SR/PCR	S	V _L	SalI
92-100	ACTAGTCGACCGA(TC)AT(TC)GT(GC)CT(GCT)AC(ACG)CA(AG)TCICCA	3SR/PCR	S	V _L	SalI
92-101	ACTAGTCGACCGA(TC)AT(TC)GT(GC)CT(GCT)AC(ACG)CA(AG)TCGCCA	3SR/PCR	S	V _L	SalI
92-102	ACTAGTCGACCGA(TC)AT(TC)GT(GC)CT(GCT)AC(ACG)CA(AG)TCGCCA	3SR/PCR	S	V _L	SalI
92-105	CACCCCATCTGTCTATCCACTTC	Probe	S	C _H 1	none
92-107	ATG(GA)A(GC)TT(GC)(TG)GG(TC)T(AC)A(AG)CT(GT)G(GA)TT	3SR/PCR	S	V _H -leader	none
92-108	ATG(GA)AATG(GC)A(GC)CTGGGT(CT)(TA)T(TC)CTCT	3SR/PCR	S	V _H -leader	none
92-109	ACTAGTCGAC(GC)AGGT(CG)(AC)A(AG)CTGCAG(CG)AGTCT	PCR	S	V _H	SalI
92-110	ACTAGTCGAC(CG)AGGTG(CA)AGCTC(CG)(AT)(AG)(CG)A(AG)(CT)C(CG)GGG	PCR	S	V _H	SalI

¹ ' indicates the presence of the T7 promoter binding site at the 5'-end of the primer.

² S (sense) refers to sequences that are homologous to the RNA, whereas AS (anti-sense) refers to the sequences that are complementary to the RNA.

Fig. 2
CONTINUED

4 / 4

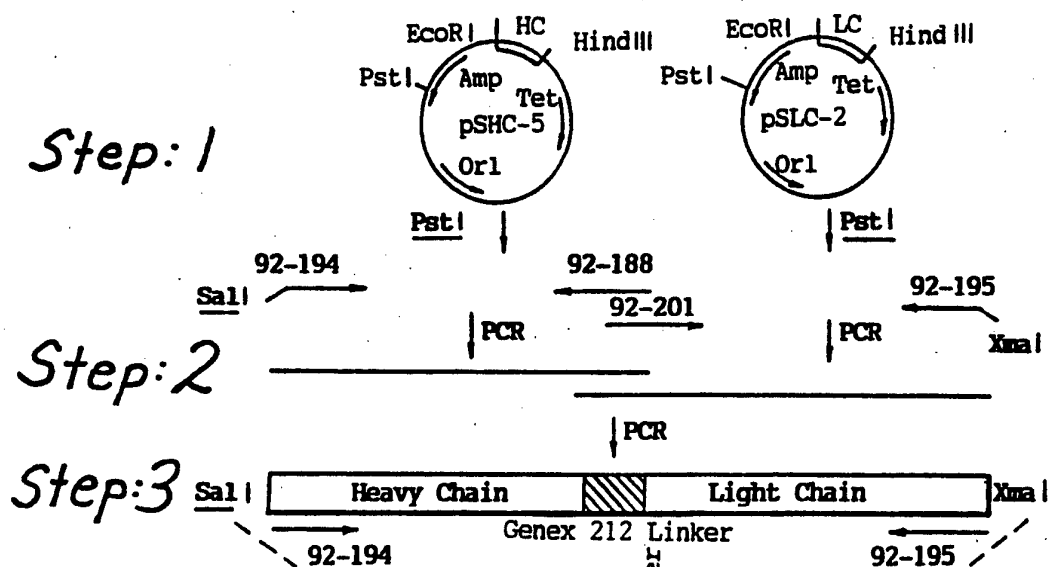


Fig. 3

