LAH 405

Chapter 13
Construction of cDNA from Single Unstimulated Mouse B Lymphocytes: Method and Application to the Study of Expressed Antibody Repertoires in FACS-Sorted Murine B Cell Subsets

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We have developed a method for generating cDNA from individual, unstimulated, FACS-sorted mouse B lymphocytes, and for recovering expressed antibody transcripts. The procedure involves staining and sorting for cell subsets of interest in the presence of RNase to remove exogenous RNA. Cells are then deposited at one per tube into lysis solution containing RNase inhibitor, proteins are denatured by heating, and cDNA is synthesized using random hexanucleotide primers. The product is aliquoted, and selective PCR amplification is performed using constant region primers and primers to the highly conserved 5′ portion of the variable region. Expressed transcripts are recovered from up to 90% of cells. Diversity of the sample and lack of contamination are confirmed by restriction enzyme-digestion of the products and by sequencing. V genes representative of 14 V_H families have been successfully amplified. Analysis of the samples yields a picture of the expressed B cell repertoire that is not biased by stimulation or transformation, and permits simultaneous recovery of more than one gene from a single cell, such as heavy chain and light chain.

Introduction

Immunoglobulin genes are assembled from discontinuous germline gene segments during early stages of B lymphopoiesis [1–3]. Heavy chain genes are assembled from a variable (V_H) element, one or more diversity (D) elements, and a joining (J_H) element; light chain genes are made by joining V_L and J_L elements. The potential diversity of the antibody repertoire derives from the multiplicity of encoded elements, from variability in their sites of joining, and from addition of non-templated nucleotides at their junctions, termed N regions [4], and of templated nucleotides, termed P sequences [5]. It is estimated that these mechanisms can generate more than 10^{12} different antigen binding sites [6, 7]. Additional diversity is generated later, during the immune response via somatic mutations of already rearranged V genes.

B cell subsets can be distinguished by FACS according to their cell surface phenotype and size and divided according to stage of differentiation, such as pro-B cell to plasma cell, and B cell lineage. It is often of interest to determine what subset of antigen combining sites from the vast potential of antibody diversity is expressed in particular populations of B lymphocytes. Ideally, a repertoire data set should include sequence of both the V_H and V_L genes and identification of the antigen. Analysis of antibody repertoires has already contributed to our understanding of immune development, immune deficiency, autoimmune disease, and genetic variations in immune response. However, there is a strong need to develop methods of repertoire analysis that are free of bias.

Most of the current information has been obtained by study of immortalized cell lines (EBV transformations and hybridomas), bulk cDNA libraries, and by hybridization techniques. It is uncertain whether cell lines and bulk cDNA are representative of the populations from which they derive. Transformation is very inefficient (from 1 in 30 to 1 in 10^7 cells), and selects cells in favored states of activation [8–11]. Similarly, while hybridomas are an excellent source of monoclonal antibodies for use in protein studies and as analytical reagents, the cell-cell fusion required for hybridoma production is inefficient and biased to certain stages of differentiation. *In situ* hybridization has recently been applied to the study of expressed V_H families and individual genes [12–15], but is not sensitive enough to detect mRNA from unstimulated cells and provides data limited by the nature of available probes, which is far less informative than a complete sequence.

Construction of conventional cDNA libraries, like cell immortalization, is limited to applications in which relatively large amounts of starting material are available. This problem has been somewhat reduced by amplifying cDNA with the polymerase chain reaction (Innis et al, 1990). Particular sets of V_H families or genes have been amplified using specific primers [16–18] and diverse multi-V_H family sets have been simultaneously amplified with degenerate primers [19–23]. Neither of these approaches is suitable for unbiased analysis of the complete antibody repertoire.

For any cDNA amplification, there remains a strong bias in favor of cells expressing the greatest amount of RNA, such as plasma cells, at the time of harvest. Moreover, PCR amplification from either bulk cDNA or genomic DNA has the problem of hybrid gene products. This artifact results from false priming by homologous gene products present in the preparation and is especially troublesome if damaged DNA is present from apoptotic cells [24, 25].

The degenerate primer sets, which have been used very successfully to capture V genes for use in expression systems, may introduce additional bias due to differences in efficiency of amplification from the cDNA by particular primer components. The linker method of Huang and Stollar [16, 26] avoids this additional bias by ligating standard primers prior to the PCR reaction, however, it is still not possible to reconstruct which heavy chains and light chains were originally associated.

Single-cell approaches

Our goal is to develop an unbiased method of antibody repertoire analysis in both mice and humans that includes complete information on both heavy and light chain and that allows

for the reconstruction of the antibody molecule. Analysis at the single-cell level addresses the limitations and biases addressed above. Here we review and detail our method for constructing cDNA from single FACS-sorted B cells and recovering expressed antibody transcripts representative of all mouse V_H families. A preliminary version has been presented elsewhere [27].

Other investigators have also emphasized the importance of performing repertoire analysis on a cell by cell basis by amplifying particular V genes from individual B cells [18, 28–30] Our approach is particularly well-suited for complete evaluation of the expressed repertoire of B cell subsets based on FACS-phenotype. It can also provide analysis of antigen-induced responses free from investigator prejudice regarding the V elements, which are "expected" to be involved.

Results and discussion

Our method, which involves preparation of cDNA from individual FACS-sorted cells, two rounds of PCR amplification, and direct sequencing of the PCR product, is diagrammed in Figure 13.1. There are several important features of this method. First, the FACS and molecular biology are well integrated. The cells are stained as usual with fluorophore-MAb conjugates to cell surface markers treated with RNase, and sorted directly into a small volume of lysis solution that contains RNase inhibitors. In standard sorting, undesired cells and sheath fluid flow straight down into an aspirator, while selected cells are charged and deflected at an angle. For the preparation of cDNA from single cells, the voltage to the deflection plates and aspirator position are adjusted so that unselected cells and sheath fluid are removed at an angle, while selected cells are sorted vertically. This ensures that the cell lands directly into the 4-μl lysis solution and improves the recovery of single-cell V_H transcripts. Sorted cells can be frozen at −70°C for at least a year before preparation of the cDNA.

cDNA preparation is the most crucial step in the procedure. Samples are processed quickly and both Placental RNase Inhibitor and Inhibit-ACE are used. Most importantly, Inhibit-ACE is stable for the short period at 65°C, which is needed to denature the proteins and free the mRNA. An important advantage of our approach is that RNA purification is not needed for single-cell cDNA. Note, however, that the method is not suitable for much larger numbers of cells. Protein from 10 to 100 cells will be enough to inhibit the reverse transcription reaction. The reverse transcription reaction is primed with random hexanucleotides, which allows for the recovery of additional transcripts, such as V_L. V_H transcripts are routinely recovered by PCR from only 1/10th of the cDNA obtained from a single cell. This allows for additional amplifications to resolve any uncertainties in the recovered sequence (possible *Taq* error, contamination, etc.). Specific primers (μ CH1 region) have also been used successfully. Since the poly-A tail is at the 3′ end of the transcript, >1 kb from the V_H region, we have avoided priming with oligo-dT.

The murine V-region primer is promiscuous

cDNA is first amplified by PCR between a Cμ oligomer and a V_H-region oligomer, MsV_HE, which represents a consensus of highly conserved codons at the beginning of framework I (see Fig. 13.1). Sequence comparison of MsV_HE with genes representative of all V_H families indicates a minimum homology of 70% and a minimum Tm of 50°C. Matching at the 3′ end is sufficient to ensure good primability. This single promiscuous primer, MsV_HE, is among the 24 primers used in the degenerate mix of Orlandi et al [22]. With an annealing temperature of 50°C, MsV_HE is successful for all 14 V_H families. There appear to be no other sites beside the beginning of FR1 that should prime with MsV_HE at this annealing temperature.

We first tested the effectiveness of the first round PCR primers against known murine V_H genes. Messenger RNA was prepared from cell lines representative of 11 V_H families (V_H1/J558, V_H2/Q52, V_H3-3660, V_H8/3609, V_H4/X24, V_H5/7183, V_H6/J606, V_H7/S107, V_H9/VGAM3/8, V_H11, and V_H12) and reverse transcribed into cDNA. V_H genes from all of these families were successfully amplified in this way (data not shown).

The recovery of V_H genes is excellent

We recover V_H transcripts from up to 90% of FACS-sorted B cells after two rounds of PCR. Figure 13.2 shows the recovery from three different FACS-sorted B cell populations. Bands are typically weak, and often not visible, after the first amplification. However, most samples yield strong V_H gene bands after the second amplification. For the samples shown here, the second PCR was done with nested primers at both the 5′ and 3′ ends ($MsC\mu E_{nest}$ and MsV_HE_{nest}). However, we now prefer to nest only at the 3′ (μ) end since this leaves more sequence information at the 5′ end of V_H.

Occasionally we observe two bands from a single-cell sample following amplification with the nested primers. According to the principle of allelic exclusion, each B cell is expected to produce only one functional heavy chain [31]. In our case double bands have arisen from second priming sites on the gene and from occasional cross-contamination. Comparison of MsV_HE_{nest} with representative V_H genes suggests such alternative priming sites. Furthermore, sequence analysis of cloned bands demonstrates that the double bands generally arise from alternative priming sites within the V_H gene (data not shown). Thus, there is no evidence that allelic exclusion is violated. We find that semi-nested amplification, with only a nested Cμ primer, often eliminates the second, smaller band. In addition, the nested V primer, MsV_HE_{nest}, does not work well on some genes such as the SM7 family members, which have a codon deletions at position 13 [32, 33], the 3′ end of the primer.

A large set of different V gene products result. Digestion of the PCR product with the restriction enzyme *Alu*I results in distinct banding patterns for all samples tested, indicating unique rearrangements (Fig. 13.2). Furthermore we have obtained distinct sequences from more than 250 different FACS-sorted single-cell samples indicating that we are truly analyzing individual B cells with unique VDJ rearrangements. It is important to include negative controls for the complete procedure. We use polystyrene beads that are routinely mixed with spleen cells and FACS-sorted based on their distinct phenotype. They are almost always negative for V_H transcripts. False positives can be attributed to handling errors.

The sequences from FACS-sorted B cells represent 13 different V_H gene families, including three, V_H10 (DNA4), V_H14 (SM7), and V_H13 (3609 N), which were not represented among the cell lines. Thus genes from 14 V_H families have been amplified from either cell lines or sorted cells. We have amplified V_H8 (3609) successfully from cell lines, but not from FACS-sorted B cells. The V_H8 family represents only 2% of the V genes in cDNA libraries made from LPS-stimulated B cells [34]. Our failure to recover

I. FACS

A. Stain

Usual staining,
Treat with RNAse

B. Bulk Sort

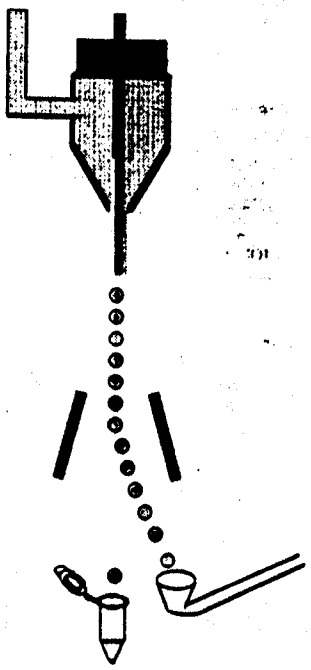
Bulk sort first, number of cells permitting

Reanalyze

C. Deposit single cells

Sort vertically to ensure cells land in the lysis solution which contains RNAse inhibitor

Freeze



II. Amplification and Sequencing Scheme

A. Prepare cDNA from mRNA

Denature proteins.
Reverse transcription with Random hexamers

B. First Amplification

PCR with Cμ and a promiscuous Vh primer

C. Second Amplification

Nested at the μ end
Targets for M13 sequencing primers can be tailed at either or both ends

D. Taq Sequencing

Dye-labeled primers

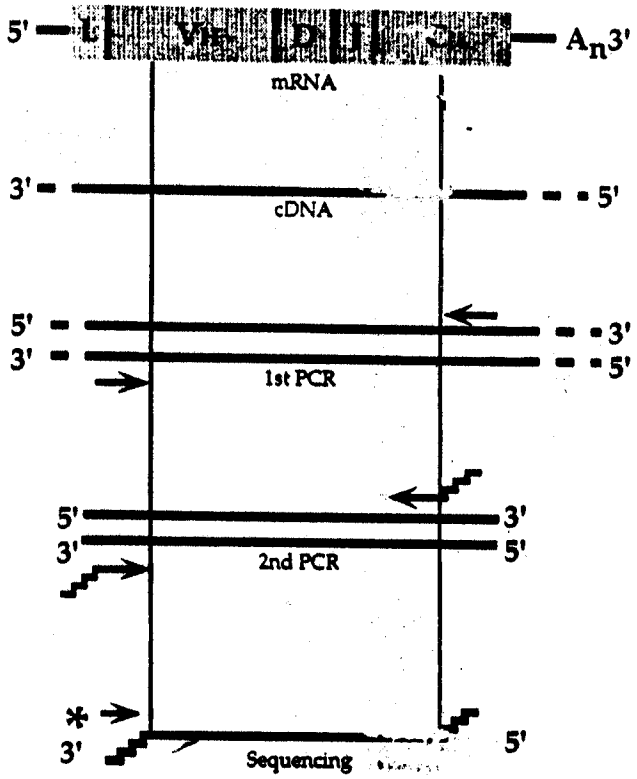


Fig. 13.1. *Analysis of expressed V_H genes from single FACS-sorted B cells.* See text for details.

these genes to date is consistent with low usage. However, some members of this family do not match MsV_HE as well as other V_H genes.

Some samples do not yield suitable PCR products after two rounds of amplification. For these negative samples, we go back to the cDNA and try again. In order to maximize successful recovery, we use more cDNA for the second attempt (1/4 of the total). In addition, we lower the annealing temperature to 44°C for the first

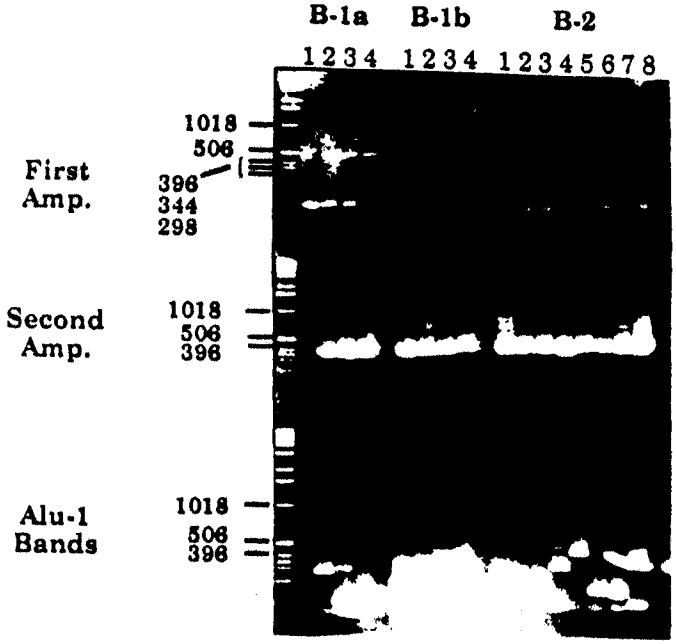


Fig. 13.2. *Murine peritoneal cells were pooled from washes of 10 Balb/cHz mice (female, age 5.5 months).* Mouse peritoneal cells were stained with and sorted into three subsets as described previously [44, 45]: representative samples are shown on agarose gels after the first and second amplifications and after digestion with *AluI*. Overall recovery for this data set was 86%. For the 27 B-1a cell samples, a few faint bands were seen after the first amplification and 22 bands were observed after the second amplification (recovery = 81%). For the 28 B-1b cell samples, 12 to 14 faint bands were seen after the first amplification and 27 bands were observed after the second amplification (recovery = 96%). For the 29 B-2 cell samples, 5 to 7 faint bands were seen after the first amplification and 23 bands were observed after the second amplification (recovery = 79%). Sequences were recovered from all 72 samples.

few cycles to improve the chance of recovering unknown genes that might not match MsV_HE well. Our experience suggests that the occasional failure to recover transcripts is due to isolated mistakes before or during the cDNA preparation rather than failure during the amplification procedure.

Analysis of the PCR products

A major difficulty in the study of the V gene repertoire is the effort required to clone and sequence the genes. In order to produce a data set rapidly, we prefer to sequence directly from the PCR products. Sequencing primer sites (designated m13 and m13r) are tailed onto the V_H product during the second amplification. These primer sites correspond to stock Dye-labeled primers available for fluorescence-based sequencing from Applied Biosystems. However, we can also incorporate *Eco*RI restriction sites into the upstream and downstream primers for direct cloning of the PCR product. This is particularly useful when doublets need to be evaluated or when the gene product is to be expressed with light chain to form a functional antibody.

Conclusions

We have developed a robust method for amplifying V genes from single, unstimulated B cells that is free of the biases imposed by earlier approaches. Although the method is somewhat time consuming and demanding, we feel the resultant data are well worth the effort. Once the method is mastered, it is reasonable for an individual to amplify and sequence 100 samples per month. Finally, we anticipate the methodology will be extended to include light chain amplification and the expression of functional protein.

Materials and methods

General precautions to avoid RNA cross-contamination or loss

Great care is taken throughout the procedure to ensure successful preparation and amplification of the cDNA without cross-contamination. All equipment (pipetmen, racks, etc.) and work surfaces are cleaned first with 0.1 M NaOH and then 95% EtOH. Aerosol-resistant pipette tips are used. Tubes are opened in isolation and with fresh gloves. Whenever possible manipulations are performed in a hood, with a UV light source; however this is not a requirement. RNase-free solutions are used for all cDNA preparation and amplification.

FACS

Preparation of single-cell suspensions and staining are done as usual, with a staining media consisting of deficient RPMI (No phenol red, flavins, etc.) 10 mM Hepes and 3% newborn calf serum, 0.2% sodium azide. Propidium iodide is added to exclude dead cells [35]. After staining, cells (2.5×10^8/ml) are incubated with 10 ng/ml RNase A (Boehringer Mannheim, Indianapolis, IN) for 30 minutes on ice and then diluted 10-fold (2.5×10^7 cells/ml, 1 ng/ml RNase) for sorting.

Cells are sorted on "Flasher," an extensively modified dual laser FACS II equipped with appropriate filters for four-color immunofluorescence [36] (Becton Dickinson, San Jose, CA) and interfaced to a VAX 6300 computer (Digital Equipment, Maynard, MA) running FACS/Desk software (W. Moore, Stanford University). Machine calibration with standard fluorescent polystyrene microspheres (Spherotech, Libertyville, IL) and fluorescence compensation is done with FACS/Shiva software (M. Bigos, Stanford University). Prior to sorting for construction of cDNA from single cells, the fluidics system is washed with 0.1 M NaOH for five minutes to remove extraneous nucleic acid and 70% ethanol for five minutes to sterilize. Sheath fluid is deficient RPMI, with 10 mM Hepes and 0.5 ng/ml RNase. Cells are kept at 4°C before, during, and after sorting.

Sample deposition and cell lysis

The B cell populations are bulk sorted first and then reanalyzed to check purity. Polystyrene beads (2.2 μ, 3×10^6/ml; Spherotech), which can be readily distinguished from lymphocytes by forward scatter, are added to a sample of the cell suspensions as a negative control. One cell or bead is deposited by FACS per 0.5 ml microcentrifuge tube containing 4 μl of lysis buffer (0.5 × PBS; 10 mM DTT; 2 U/μl placental RNase inhibitor, and 0.2 U/μl Inhibit-Ace, 5 Prime → 3 Prime, Boulder, CO). Tubes are kept on ice prior to sorting and on a cold stage during the sort. In standard sorting, unwanted cells and sheath fluid flow straight down into an aspirator, while selected cells are charged and deflected at an angle. For the production of cDNA from single cells, the voltage to the deflection plates and aspirator position are adjusted so that unselected cells and sheath fluid are removed at an angle, while selected cells are sorted vertically. Tubes are aligned so that the cell lands directly into the 4 μl of lysis buffer. Four to six cells are

Table 13.1. Primers used in amplification of mouse V_H cDNA[a]

Primer	Nucleotide sequence	Bases
MS V_HE	GGGAATTCGAGGTGCAGCTGCAGGAGTCTGG	31
MsCμE	ATGGCCACCGAATTCTTATCAGA	23
m13MsCμN	TGTAAAACGACGGCCAGTCATTTGGGAAGGACTGA	35
MsV_HN	GGGAATTCTGGACGAGACTTGGTGCAGC	28
m13rMs V_HN	CAGGAAACAGCTATGACCTGGACGCTTGGTGCAG	34

[a] *Eco*RI cloning sites are underlined (e.g., GAATTC). Targets for sequencing primers are double underlined. The V_H primers match the beginning of framework 1 (Ms V_HE-codons 1-7.7; Ms V_HN codons 7-14). Ms V_HE and MsCμE are used for the first amplification. Several primer options are available for the second amplification. MsV_HE and m13MsCμN have been the preferred choice.

processed (sorted, spun, frozen on dry ice) at a time. Samples can be stored at −70°C before preparation of cDNA for at least one year.

Construction of cDNA

Samples are kept on dry ice while primer mix is added. Each added 7-μl aliquot contains: 1 μl random hexamer (stock at 300 ng/μl), 1.0 μl of 10% NP-40 (Boehringer Mannheim), 1 U of Inhibit-ACE (1 to 2 μl, concentration from supplier varies, 5 Prime -> 3 Prime Inc., Boulder, CO) and RNase-free water (BioWhittaker, Walkersville, MD) to make a total of 7 μl). After the primer mix is added to all tubes, samples are placed in a thermocycler that has been preheated to 37°C, heated to 65°C for one minute, then cooled to 10°C for at least three minutes. Samples are removed from the thermocycler one tube at a time and 14 μl of reverse transcriptase (RT) mix is added immediately (final cDNA volume is 25 μl). The RT mix consists of 5 μl of 5× RT buffer, 1 μl of stock solution that is 25 mM for all four dNTP's, 2 μl of 100 mM DTT, RNase-free water sufficient to bring the final volume to 14 μl, 1.0 μl rRNAsin (10 U/μl, Gibco BRL), 1 U of Prime Inhibit-Ace (1 to 2 μl), and 1.5 μl RT (200 U/μl, Gibco-BRL Superscript II). Solution is kept at room temperature for 10 to 15 minutes to promote annealing, then 37°C for 30 minutes, then heated to 90°C for six minutes to destroy enzyme, then cooled to 4°C. The cDNA solution may be aliquoted and stored at −20°C until ready for amplification.

Cell lines

The following cell lines (kindly provided by Michael Cancro, Ed Voss, Joan Goverman, Richard Hardy, and Steve Clarke) were used to test the V_H primes: Murine lines are J558 (J558 family, ATCC #TIB6); Nab2 (3609 family [37]; B12.12E11H3, G9 (3609 family; Cancro, unpublished); 4-4-20 (J606 family, [38]); 3A4 (Vgam3.8 family); B7.14D1, B5, A7 (Vgam3.8 family; Cancro, unpublished); MOPC315 (36 to 60 family, ATCC #TIB23); F12-75-13 (X-24 family; Cancro, unpublished); S107 (T15 family, [39]); 18-2-3 (Q52 family, [40]); P3x+78 (7183 family, [41]; 2C8 (VH11 family, [42]); CH27LX.1FG (VH12 family [43]).

First amplification of specific cDNA

cDNA is amplified by the polymerase chain reaction [44] between a promiscuous 5′ V_H primer and a Cμ primer (primers, which were prepared at the Stanford Protein and Nucleic Acid Facility, are shown in Table 13.1). The cDNA (1/10 to 1/2 of the sample) is added to a primer mix consisting of 1× *Taq* buffer, 2 mM $MgCl_2$, 100 ng of both the upstream and downstream primers, and an appropriate amount of RNase-free water. Final sample volume is 98 μl. Samples are heated to 96°C for three minutes to unfold the DNA, then cooled to 50°C. Two microliters of *Taq* mix (0.4 μl of *Taq* at 5 U/μl from Gibco BRL or other source, 1.0 μl of dNTP stock, 25 mM of each, and 0.6 μl of 1× *Taq* buffer) is added to two to four samples at a time, layered with mineral oil, and then returned to the thermocycler (50°C). Samples are amplified for 35 cycles: 97°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. Samples are purified with QiaQuick PCR spin columns (Qiagen, Chatsworth, CA) using 50 μl of 10 mM Tris, pH 7.2, for the final elution. EDTA is avoided since it can interfere with the *Taq* sequencing.

Semi-nested amplification

The second amplification, which is semi-nested, uses an internal constant region primer and the same promiscuous V-region primer. We have also used an internal promiscuous V_H primer (Table 13.1); however, we have stopped using this primer because V_H information (the first seven codons) is lost. An aliquot (5 to 8 μl) of the purified first-round PCR product is added to a primer mix that is same as above except for the primers, heated to 96°C for three minutes, and cooled to 50°C. *Taq* mix (same as above) is added and the samples amplified as above. The primers for the second amplification can incorporate *Eco*RI sites for cloning or M13 sequence for *Taq* cycle sequencing. Positive samples are identified by ethidium bromide staining in agarose gels.

Sequencing

Positive samples are purified with QiaQuick PCR spin columns (Qiagen) using 50 μl of 10 mM Tris, pH 7.2, for the final elution. PCR products are sequenced directly using Dye-labeled primer chemistry (Applied Biosystems).

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