Retrieval of DNA Sequences Present at an Extremely Low Frequency

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ABSTRACT

We describe a method for retrieving sequences with one or two point mutations of a given target sequence, which are present in a DNA population at a frequency of 1 in 466 ´ 10^3 and 1 in 28 ´ 10^3 molecules, respectively. By stringent hybridization to a stable, chemically immobilized probe, a large excess of unrelated fragments is removed, and the bound sequences are dissociated and amplified. By repeating the hybridization-amplification cycles twice, we achieved an estimated enrichment of 404 000-fold and 1612-fold, respectively, which was confirmed by cloning the resultant products and sequencing 35 clones. This procedure can be applied to retrieve mutated sequences that exist at an extremely low frequency in a DNA population.

INTRODUCTION

A fundamental problem frequently faced in molecular biology is the identification and isolation of rare, mutated molecules present in a given DNA population by utilizing existing sequence data. Examples of such cases include germ-line mutations as in genetic disease (3,5), differences acquired by infection with a DNA-based pathogen (18), somatic mutations that lead to cancer development (7,9) and, particularly, natural mutations occurring during the immune response (4,6,14). Molecular studies using conventional hybridization techniques are hampered by the large repertoires and the high degree of homology, at the DNA level, among antibody molecule families with the same specificity. Analysis of the repertoire by cloning is limited by the transformation efficiency of the host organisms, in addition to the problems of handling and screening such large libraries.

We have recently described a simple and reliable method of direct chemical immobilization of DNA probes to ordinary polystyrene microwells, allowing both amplification and hybridization to take place in the same well (13). We reasoned that this approach could be particularly applicable for enrichment and retrieval of rare DNA sequences, suitable for somatic mutation studies. In the present investigation, we report *(i)* definition of the conditions for achieving effective retrieval and *(ii)* evaluation of the method by retrieving extremely rare DNA fragments containing point mutations of a target nucleotide sequence from a large pool of synthetic DNA molecules with great sequence homology.

MATERIALS AND METHODS

Oligonucleotides

We designed and synthesized the oligonucleotides as described in Table 1.

Oligonucleotide Immobilization

The CDR3 oligonucleotide was added a 5′ amino group and a methylene spacer for the immobilization reaction. Oligonucleotide immobilization on polystyrene surface of enzyme-linked immunosorbent assay (ELISA) wells (MaxiSorp™; Nalge Nunc International, Roskilde, Denmark) was performed utilizing carbodiimide chemistry as described elsewhere (13). Briefly, 50 pmol of CDR3 oligonucleotide diluted in 100 µL of a solution containing 0.5 mg/mL *p*-nitrophenol and 0.5 mg/mL 1-ethyl-3-3-(3-dimethyl-aminopropyl) carbodiimide (EDAC) were transferred to each microwell. After a 2-h incubation with continuous shaking, in the dark at room temperature (RT), excess reagents were removed, and the wells were washed four times with wash buffer (0.1% Tween® 20 and 1 M NaCl). The wells were then blocked with 100 µL of blocking solution (0.5% bovine serum albumin [BSA], 0.1 M NaCl and 10 mM PO_4^{3-}, pH 7.4) for 30 min at RT. After a second wash step, (four times with wash buffer and twice with deionized water), the wells were stored at -20°C until needed.

Hybridization Conditions

As source of the complementary strand, we used a gel-purified polymerase chain reaction (PCR) product (250 bp) of the primers [CDR3] and [FR1] from a cDNA mixture of a polyclonal B-cell population. Labeling with

Table 1. Oligonucleotides

Primers for the generation of the PCR product containing the CDR3 sequence were selected from the sequence of the human recombinant antibody fragment specific to the V3 region of the human immunodeficiency virus Type 1 (HIV-1):

FR1: 5′-GTCCTCGCAACTGCCCCATGGCCCAGGTGCAGCTGCACC-AGTCGGG-3′

CDR3: 5′-CTCCGGTACCTATTGGCTATAGGTGCG-3′

Degenerate CDR3 oligonucleotide: 5′-GAAGGAGCCACCCCACAAGCGNANN-TATANNNAATANNTANNNNAGGGGGGGACATCAAGCAGCC-3′

Primers for the amplification of the degenerate CDR3 oligonucleotide:

HP1: 5′-GAAGGAGCCACCCCACAAG-3′

HP2: 5′-GGCTGCTTGATGTCCCCCC-3′

Sequencing primers:

T3: 5′-AATTAACCCTCACTAAAGGG-3′

T7: 5′-GTAATACGACTCACTATAGGGC-3′

[^{32}P]dCTP and dATP (Amersham International, Little Chalfont, Bucks, England, UK) was performed by nick-translation (11) to a specific activity of 1.5×10^8 counts per minute (cpm) per µg DNA. Hybridization experiments were carried out as follows: the radiolabeled [FR1-CDR3] product was diluted in 6× standard saline citrate (SSC) buffer to a final volume of 100 µL and transferred to the coated microwells; the aqueous phase was covered with paraffin oil to avoid evaporation. The entire hybridization procedure was performed on a thermal cycler modified to accept ELISA wells (I.H. block; Genesys Instruments, Cambridge, England, UK). After a heat-denaturation step (92°C, 5 min), the wells were incubated at the desired hybridization temperature for 30 min. The solution was discarded, and the wells were washed four times with 300 µL of preheated 6× SSC solution at hybridization temperature to remove unreacted DNA sequences. Specifically hybridized molecules were released by heating the wells to 92°C for 3–4 min in 100 µL 6× SSC. Radioactivity of the resultant supernatant was measured by liquid scintillation using a Model LS 1701 Beta-Counter (Beckman Instruments, High Wycombe, Bucks, England, UK).

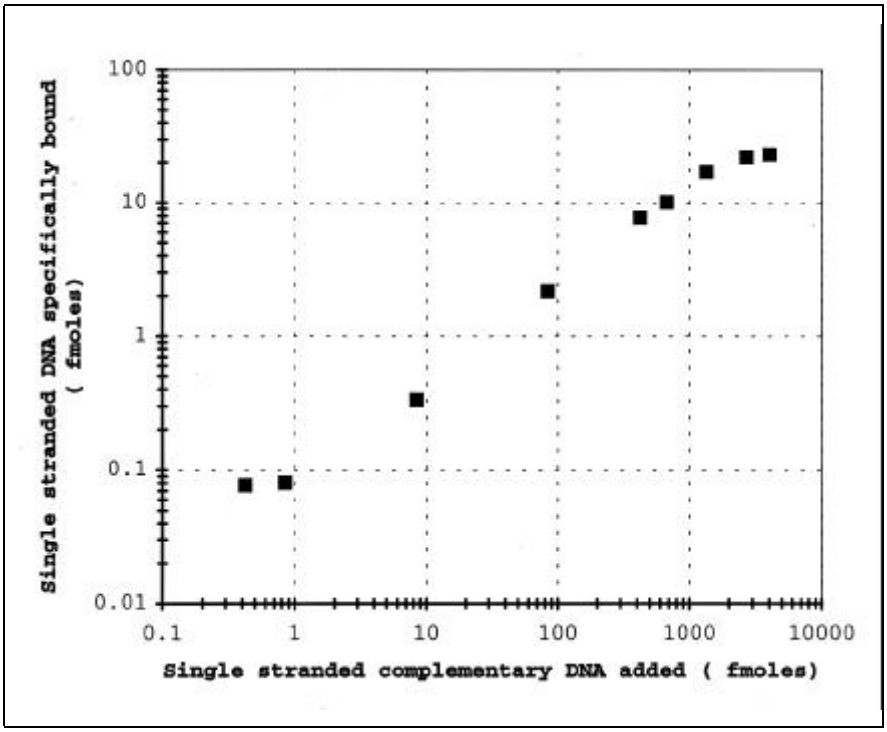

Figure 1. Hybridization capacity of the immobilized probe. As source of the complementary strand, a gel-purified radiolabeled PCR product of the primers [CDR3] and [FR1] was used. Labeling with ^{32}P was performed by nick-translation to a specific activity of 1.5×10^8. Hybridization was done at 55°C for 30 min. Hybridized molecules were released by heating the solid phase at 92°C for 3 min in 6× SSC, and the resultant radioactivity was quantitated by liquid scintillation.

Screening of the Synthetic CDR3 Library

The conditions applied for each hybridization-amplification cycle were as follows: hybridization solution contained 6× SSC, 0.5% sodium dodecyl sulfate (SDS), 100 ng/mL salmon sperm DNA, and the wash buffer contained 2× SSC, 0.5% SDS. Ten nanograms of the degenerate CDR3 oligonucleotide were allowed to hybridize at 60°C, as described above in both [CDR3]-coated and BSA-blocked wells. After washing 4 times with wash buffer and 4 times with 1× PCR buffer (Stratagene, La Jolla, CA, USA), 100 µL 1× PCR buffer were added, and the wells were heated to 92°C for 3 min to recover bound molecules; PCR amplification followed using 1 µM of each primer [HP1 and HP2], 0.2 mM dNTPs and 2.5 U AmpliTaq® (Perkin-Elmer, Norwalk, CT, USA) per reaction. Cycling was done on Cetus® PCR Apparatus (Perkin-Elmer) between 92°, 54° and 72°C for 35 cycles. Two more hybridization-PCR steps were performed at 65° and 70°C, respectively.

Cloning and Sequencing

The PCR fragment was treated with Klenow and ligated in the *Sma*I site of Bluescript® plasmid (Stratagene). Sequencing was performed in both strands using the standard T3 and T7 primers (shown above), the Sequenase® Version 2.0 Kit (Amersham International) and the producers protocol.

RESULTS AND DISCUSSION

Covalent immobilization of the 5′ aminated [CDR3] oligonucleotide, identical to a specific CDR3 hypervariable region of an immunoglobulin variable region clone (1), was performed after carbodiimide activation of the polystyrene surface of ELISA wells; a hexamethyl diamine spacer was also included to enhance molecular flexibility. We investigated the hybridization capability of the immobilized probe by adding different amounts of complementary DNA. As a source of the complementary strand, a gel-purified [FR1-CDR3] radiolabeled PCR product (250 bp) was used. The maximum capacity of the wells, under the conditions applied, was approximately 25 fmol (Figure 1).

The observed plateau of the amount of DNA annealed at higher concentrations was related either to the high rate of self annealing of the complementary DNA or due to saturation of the available annealing sites on the solid phase. Hybridization efficiency in relation to soluble single-stranded DNA added, ranged between 0.5% and 7%, in agreement with previous reports (10). Nonspecific binding of DNA to the solid phase was below 15 amol. Hybridization of 2 ng (15 fmol) of [FR1-CDR3] radiolabeled PCR product was carried out at 40°, 45°, 50°, 55° and 60°C to immobilized CDR3 to define the conditions for maximal specific binding. Hybridization with minimal nonspecific binding stabilized at and above 50°C. The specificity of the interaction between the immobilized oligonucleotide and the complementary DNA was evaluated by adding excess unlabeled, nonspecific, competitor DNA in the hybridization solution. Salmon sperm DNA, cut with *Eco*RI, was added in molecular excess up to 80 000 times. Only a moderate reduction in the hybridization efficiency was observed, indicating the considerable selective power of our system.

The efficiency of our method to selectively retrieve rare DNA sequences was evaluated. A 67-base oligonucleotide was designed containing the sequence of the CDR3 region of a recently characterized recombinant human anti-V3 (HIV-1) antibody fragment (1) with 12 degenerate bases and a constant priming site at each end. The artificial population produced was designed to simulate usual situations of a given library or a cDNA population, with an estimated 16.7×10^6 unique sequences present. The degenerate oligonucleotide was subjected to three selection cycles, with each cycle consisting of hybridization of the DNA sequences to immobilized amino-CDR3, removal of the unhybridized material, detachment of the CDR3-hybridized sequences by denaturing at 92°C and amplification of the resultant sequences by PCR (Figure 2). After the first selection cycle with a hybridization temperature of 60°C, PCR products were present from both the CDR3-coated wells and the negative control wells (Figure 3A). This probably also reflects amplification of DNA molecules bound nonspecifically to the polystyrene surface. When two further selection cycles with increasing hybridization stringency conditions (at 65° and 70°C, respectively) were performed, the nonspecific binding decreased after the second cycle (Figure 3B) and disappeared at the end of the third cycle (Figure 3C). The final PCR product was gel-purified, and a portion was re-hybridized back to the selection well for confirmation of the specificity of the product. It gave a significantly higher and specific hybridization signal as compared with the original degenerate oligonucleotide. The PCR product from the last hybridization was also cloned and sequenced in both strands. Sequencing results showed that 30 out of 35 clones contained only one nucleotide difference (GC to AT) from the selection sequence. Of the remaining five clones, three had two differences, one had 9 and one had 10. The resultant sequences were in agreement with theoretical calculations based on the melt-

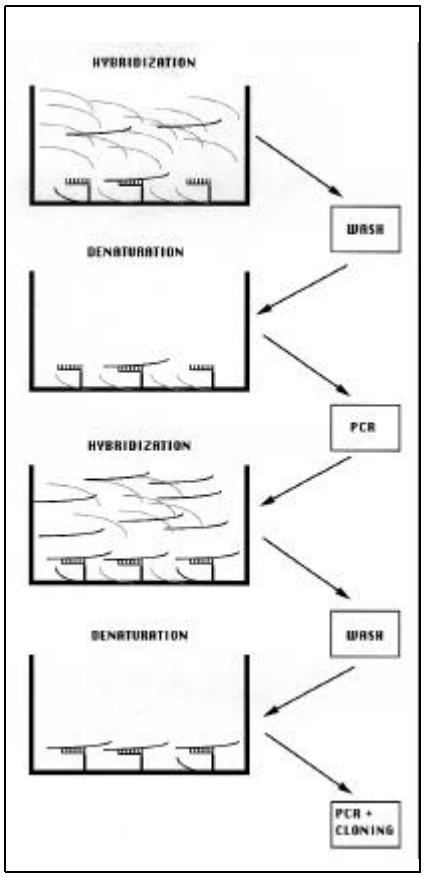

Figure 2. Schematic representation of the retreival procedure from the synthetic library of oligonucleotides.

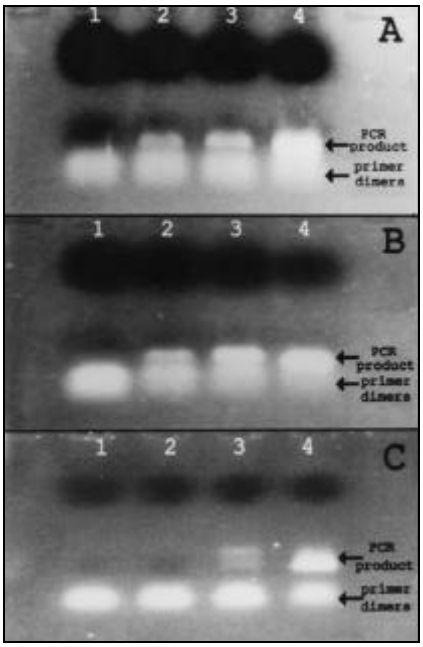

Figure 3. Analysis of PCR products by agarose electrophoresis after (A) one, (B) two and (C) three retrieval cycles. The first cycle consisted of hybridization of the degenerate CDR3 oligonucleotide, recovery and PCR amplification of the bound molecules. 10 ng of the PCR product were subjected to the second retrieval cycle. Similarly, 10 ng of the resultant PCR product were further subjected to the third retrieval cycle. Lane 1, negative PCR control; lane 2, PCR products after the retrieval procedure in CDR3-negative wells; lane 3, PCR products after the retrieval procedure in CDR3-coated wells; lane 4, positive PCR control, containing 1 ng degenerate CDR3 oligonucleotide.

ing point temperatures (Oligo™ Version 4.01; National Biosciences, Plymouth, MN, USA), which, according to the software, 70°C should permit maximum mismatch of 2 nucleotides (nt). We considered the clones with 9- and 10-nt differences as the background level, since these molecules constituted the majority (over 60%) of the original population. All the nucleotide differences observed were located entirely on the degenerate positions of the oligonucleotide, which argues against the possibility of DNA polymerase errors. Taking into account the fact that the theoretical frequency of molecules with 1 nt difference in the original population was 1 in 466 000, our method enriched the sequences with 1 nt difference 404 000 times (Figure 4). Similarly, we achieved 1612-fold enrichment of the sequences with two point mutations. Note that the main objective of the present study was not to obtain the fully complementary molecule but to enrich and retrieve sequences with one or two point mutations from the complementary sequence.

In principle, the extremely high selective power of our system, as demonstrated using an [FR1-CDR3] 250-bp PCR product of a human IgM/IgG cDNA mixture in the presence of up to 80 000 times molecular excess of irrelevant DNA, should enable this method to be applied in the retrieval of very rare DNA fragments. The method is particularly useful for studying one or more mutations of a given sequence, as is the case with the generated DNA repertoires during the process of somatic mutations of activated B lympho-

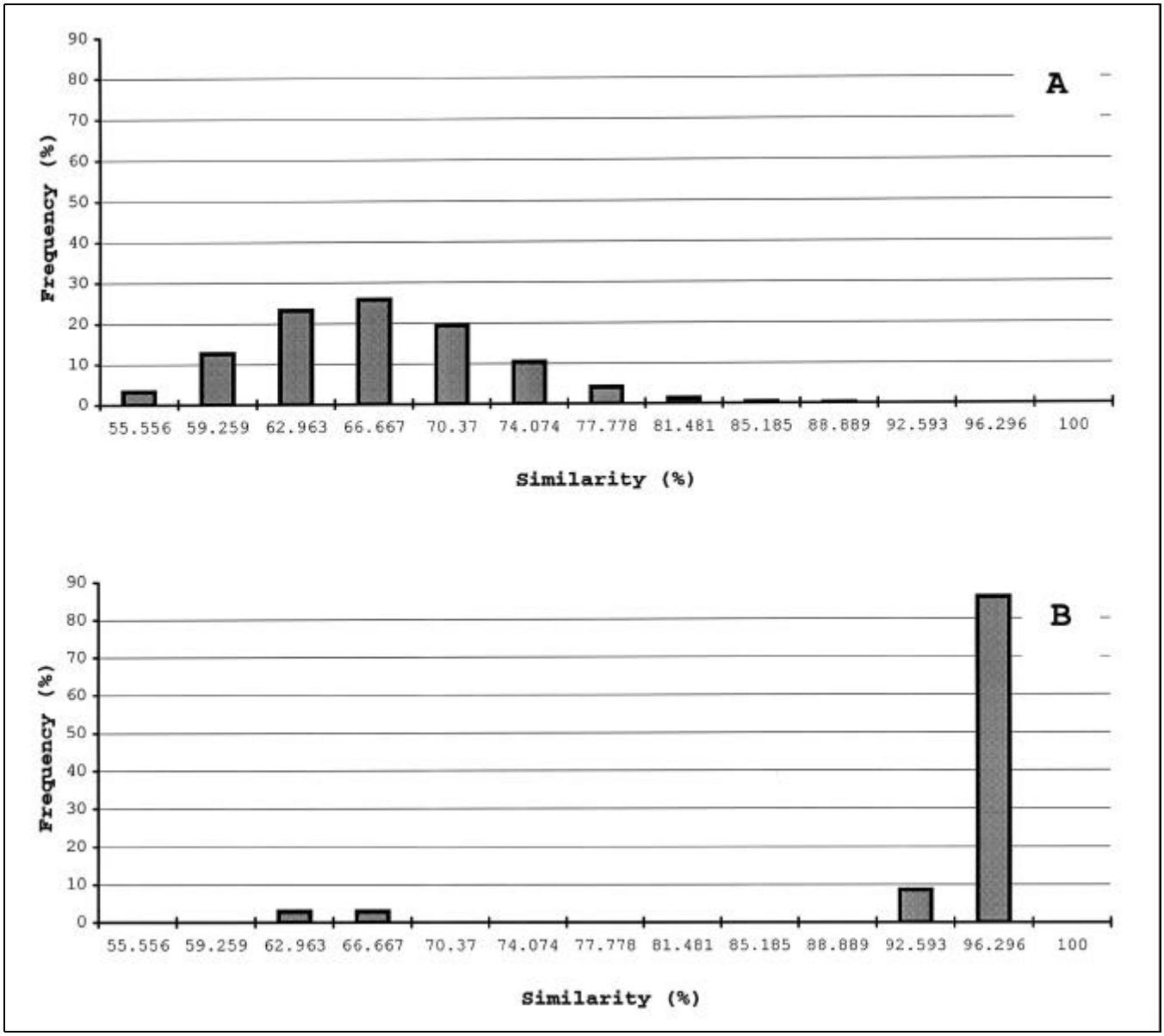

Figure 4. Comparison of sequence heterogeneity in a DNA population before and after three retrieval cycles. (A) Theoretical frequency of sequences derived from the synthesis of a 27-base oligonucleotide containing 12 degenerate bases, in relation to the homology with a given target sequence, expressed as % similarity. (B) Frequency of sequences after three retrieval cycles, as determined by sequencing of 35 different clones.

cytes. We simulated such a repertoire with an artificial degenerate oligonucleotide. The enrichment achieved was at least three magnitudes greater than by any previously reported method (2,8,12,15–17), and it could be improved even further by incorporating more cycles. Application of the procedure to a cDNA library derived from an actual antibody repertoire could result in an even more efficient retrieval since the sequence homology in a natural repertoire is expected to be considerably lower as compared to the synthetic DNA population we used, thus improving even further the selective discrimination in favor of the target molecules. Finally, note that from preliminary data, the immobilized probe can be used to retrieve plasmid DNA directly, thus dissociating it from the PCR technique and widening the range of potential applications.

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REFERENCES

1. **Chin, L.-T., A.-C. Malmborg, K. Kristensson, J. Hinkula, B. Wahren and C.A.K. Borrebaeck.** 1995. Mimicking the humoral immune response in vitro results in antigen specific isotype switching supported by specific autologous T helper cells: generation of human HIV-1 neutralizing IgG monoclonal antibodies from naive donors. Eur. J. Immunol. *25*:657-663.
2. **Kim, N.S. and S. Kato.** 1994. In-tube cDNA cloning method using a biotinylated RNA Probe. Anal. Biochem. *217*:20-24.
3. **Landergren, U., R. Kaiser, C.T. Caskey and L. Hood.** 1988. DNA diagnostics—molecular techniques and automation. Science *242*:229-237.
4. **Maizels, N.** 1995. Somatic hypermutation: how many mechanisms diversify V region sequences? Cell *83*:9-12.
5. **Martin, J.B.** 1987. Molecular genetics: applications to the clinical neurosciences. Science *238*:765-772.
6. **Neuberger, M.S. and C. Milstein.** 1995. Somatic hypermutation. Curr. Opin. Immunol. *7*:248-254.
7. **Ponder, B.** 1988. Cancer. Gene losses in human tumors. Nature *335*:400-402.
8. **Rounds, D., M. Brueckner and D.C. Ward.** 1995. Isolation of murine telomere-proximal sequences by affinity capture and PCR. Genomics *29*:616-622.
9. **Sager, R.** 1989. Tumor suppressor genes: the puzzle and the promise. Science *246*:1406-1412.
10. **Saiki, R.K., P.S. Walsh, C.H. Levenson and H.A. Erilich.** 1989. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc. Natl. Acad. Sci. USA *86*:6230-6234.
11. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, p. 10.8. 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
12. **Tagle, D.A., M. Swaroop, M. Lovett and F.S. Collins.** 1993. Magnetic bead capture of expressed sequences encoded within large genomic segments. Nature *361*:751-753.
13. **Tavernarakis, N., G. Hatzidakis, G. Vlatakis and E. Krambovitis.** 1995. Amplification and non-isotopic detection of specific DNA sequences in a single microtitre well. Serodiagnosis Infect. Dis. Immunother. *7*:202-206.
14. **Tomlinson, I.M., G. Walter, P.T. Jones, P.H. Dear, E.L. Sonnhammer and G. Winter.** 1996. The imprint of somatic hypermutation on the repertoire of human germline V genes. J. Mol. Biol. *256*:813-817.
15. **Tsurui, H., E. Hara, K. Oda, A. Suyama, S. Nakada and A. Wada.** 1990. A rapid and efficient cloning method with a solid-phase DNA probe: application for cloning the 5′-flanking region of the gene encoding human fibronectin. Gene *88*:233-239.
16. **Tsurui, H., N. Kim, Y. Umezawa and S. Kato.** 1992. Efficient cDNA cloning method using solid phase DNA probe. Nucleic Acids Symp. Ser. *27*:155-156.
17. **Tsurui, H., Y. Kumazawa, R. Sanokawa, Y. Watanabe, T. Kuroda, A. Wada, K. Watanabe and T. Shirai.** 1994. Batchwise purification of specific tRNAs by a solid-phase DNA probes. Anal. Biochem. *221*:166-172.
18. **Wieland, C., G. Bolger, G. Asouline and M. Wigler.** 1990. A method for difference cloning: gene amplification following subtractive hybridization. Proc. Natl. Acad. Sci. USA *87*:2720-2724.

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