J Korean Med Sci 1998; 13: 395-9
ISSN 1011-8934

Detection of t(11;22)(q24;q12) Translocation of Ewing's Sarcoma in Paraffin Embedded Tissue by Nested Reverse Transcription-Polymerase Chain Reaction

Ewing's sarcoma is a poorly characterized malignant tumor with a relatively uniform histologic appearance, made up of densely packed small cells with round to oval nuclei, without distinct cell borders and without any structural differentiation. Often the diagnosis has to be made by exclusion. Recently, it has been made possible to identify characteristic chromosomal rearrangements associated with certain solid tumors. More than 85% of Ewing's sarcoma and peripheral neuroectodermal tumor present a specific t(11;22)(q24;q12) balanced translocation, resulting in the production of a novel chimerical *EWS/FLI*-1 message. Using oligonucleotide primers derived from EWS and FLI-1 complementary DNAs, we were able to use reverse transcription-polymerase chain reaction (RT-PCR) as a diagnostic tool. The described nested RT-PCR method as another supportive diagnostic method enables pathologists to differentiate small blue cell tumors not only to make correct diagnosis but also to investigate retrospective archival tumor samples, using formalin fixed paraffin embedded tissue as a source of RNA.

Key Words : *Sarcoma, Ewing's; Translocation, genetic, t(11;22)(q24;q12); Nested RT-PCR*

Yong-Koo Park, Sung-Gil Chi, Hye Rim Park,* Moon Ho Yang, K. Krishnan Unni†

Departments of Pathology, College of Medicine, Kyung Hee University, and Hallym University,* Seoul, Korea
Department of Pathology, Mayo Clinic, Mayo Foundation, Rochester, MN†

Received : December 24, 1997
Accepted : March 9, 1998

Address for correspondence
Yong-Koo Park, M.D.
Department of Pathology, College of Medicine, Kyung Hee University Hospital, 1 Hoeki-dong, Dongdaemoon-gu, Seoul 130-702, Korea
Tel : +82.2-958-7842, Fax : +82.2-957-0489
E-mail : damia@chollian.net

INTRODUCTION

Ewing's sarcoma comprises approximately six to ten percent of biopsied primary malignant bone tumors and is the fourth most common primary malignancy of bone, following myeloma, osteosarcoma and chondrosarcoma (1), presenting as an aggressive osteolytic tumor with a marked propensity for dissemination. Accurate and rapid diagnosis is essential for clinical management, but classification of the neoplasm can be difficult because the microscopical appearance of the tumor is not specific. Ewing's sarcoma is thought to be the least differentiated group of small cell neoplasms with varying degrees of neuroectodermal differentiation (1). The presence of large amounts of intracellular glycogen is not a specific finding, since up to 35 percent of Ewing's sarcoma do not contain detectable glycogen, whereas many other childhood tumors do contain it (2). The MIC2 antigen, a membrane protein of unknown function, is a promising new marker (3-5). However, its presence in several unrelated types of tumors limits its reliability (3-6). Multiple cytogenetic studies of Ewing's sarcoma, beginning with the works of Aurias et al. (7) and Turc-Carel et al. (8), have clearly documented a characteristic t(11;22)(q24;q12) chromosomal translocation in the cells of Ewing's sarcoma. Approximately 90 to 95 percent of Ewing's sarcoma of skeletal and extraskeletal origin are associated with a t(11;22)(q24;q12) balanced translocation (7, 9-11). Cytogenetically identical translocations have been observed in other small round cell tumors, such as peripheral neuroectodermal tumor, peripheral neuroepithelioma and Askin tumor and small cell osteosarcoma (12).

As a consequence of this t(11;22)(q24;q12) translocation, the carboxy terminus of the *EWS* (for Ewing's sarcoma) gene from chromosome 22q12, which normally contains a RNA binding domain, is replaced by a DNA binding transcription factor termed *FLI*-1 from chromosome 11q24. The recent molecular characterization of the Ewing's sarcoma t(11;22) translocation showed that most chromosome 22 breakpoints were clustered within a small 7 kb region and a larger 40 kb region within the *FLI*-1 gene on chromosome 11 (13).

In 1992, Dellatre et al. (14) and Zucman et al. (13) cloned the breakpoint region, so molecular genetic ap-

proaches for the detection of the t(11;22)(q24;q12) translocation are now possible. Several studies reported the amplification of the generated fusion transcript, *EWS/FLI*-1, by reverse transcription-polymerase chain reaction (9, 14-16). Until now detection of the *EWS/FLI*-1 transcript by RT-PCR has been possible only in tumor cell lines established from Ewing's sarcoma patients or from tumor tissue that was immediately frozen in liquid nitrogen after biopsy. The purpose of this study was to modify the established RT-PCR protocol to detect the t(11;22) translocation in formalin fixed, paraffin embedded tissue.

MATERIALS AND METHODS

We collected ten cases from the Departments of Pathology from Kyung Hee University, Hallym University and thirty cases from the Department of Pathology, Mayo Clinic, Mayo Foundation, Rochester, MN. All the histologic slides were reviewed by two of us (YKP and HRP). We collected five, ten μm thickness paraffin sections from each case to isolate RNA. All the paraffin blocks were made after decalcification.

RNA isolation

We isolated RNA from formalin-fixed, paraffin embedded tissue samples according to the method used by Stanta and Schneider (17). In brief, tissue sections (5×10 mm) were incubated with 1 ml of xylene for 20 min. After centrifugation in a tabletop centrifuge, the pellet was washed twice with 0.5 ml of ethanol and pelleted by centrifugation. After air-drying the pellet, 300 ml of lysis buffer containing 1 M guanidinium thiocyanate, 25 mM 2-mercaptoethanol, 0.5% Sarkosyl, 20 mM Tris-HCl pH 7.5, and 6 mg/ml proteinase K was added. The mixture was incubated for 6 hours at 4℃. After this incubation, the RNA was extracted once with phenol and chloroform. The aqueous supernatant was transferred to a new tube and precipitated with an equal volume of isopropanol and the addition of 2 mg of glycogen. For quantification of RNA, OD 260/OD 280 was measured and the quality was analyzed by gel electrophoresis.

Reverse transcriptase-polymerase chain reaction

One mg of total RNA was reverse-transcribed into cDNA of 20 ml using random hexamer primer (Boehringer Mannheim, Indianapolis, IN) and 200 U of MoMuLV reverse transcriptase (Gibco, BRL, Gaithersburg, MD). The quality of newly synthesized cDNA was evaluated first by amplifying GAPDH transcripts and cDNA specimens showing GAPDH positivity were chosen for further analysis.

For specific amplification of the putative *EWS/FLI*-1 junction regions, we performed a two-step PCR reaction. The oligonucleotide primers we used in this study were previously described by Dellatre et al. (14). The sequences of primers are 5'-ACTCCCCGTTGTCCCCTCC-3' (11.3) and 5'-TCCTACAGCCAAGCTCCAAGTC-3' (22.3). A hot start PCR was performed with the following cycling conditions: denaturation at 95℃ for 1 minute, annealing at 60℃ for 45 seconds, and extension at 72℃ for 2 minutes followed by a final 10 minutes extension at 72℃. After 36 cycles, 2 ml of the first PCR products were used as template for subsequent PCR reaction using the following nest primers; 5'-GGTRGATACAGCTGGCGTTGG-3' (11.3 N) and 5'-CCAACAGAGCAGCAGCTACG-3' (22.3 N). The nested-PCR was performed for 40 cycles of denaturation at 95℃ for 1 minute, annealing at 58℃ for 45 seconds and extension at 72℃ for 1 minute 30 seconds.

Fifteen ml of nest-PCR products were resolved through a 2% agarose gel electrophoresis, stained with ethidium bromide (1 mg/L in 0.5X TBE buffer), and photographed under UV light.

Direct sequencing of RT-PCR products

The dideoxy chain-termination method of Sanger was used for direct sequencing of the RT-PCR products (18). A sequenase V 2.0 Kit (Amersham, United Kingdom) was used with Sequitide^{35}(DuPont Corp., Boston, MA). Single-stranded sequencing templates were generated from the nested-PCR products by performing an asymmetric PCR with the appropriate internal primer for the sense strand or antisense strand. Uncorporated dNTP and PCR primers were removed by washing with water in a Centricon-30 concentrator (Amicon, Beverly, MA). Sequencing reaction was performed according to the manufacturer's instruction. Sequencing reaction products were separated on a 6% polyacrylamide/7M urea gel which was exposed to Kodax XAR film (Eastman Kodak, Rochester, NY) at room temperature for 3 days. Sequencing of both strands was done to confirm the identified sequence rearrangement.

RESULTS

Histologically, all the selected case showed classic Ewing sarcoma consisted of broad sheets and large nests of uniform, small, polygonal cells with scanty pale cytoplasm and indistinct cell borders. The nuclei were round to oval with finely dispersed chromatin pattern (Fig. 1). Also noted were diastase sensitive periodic Acid Schiff

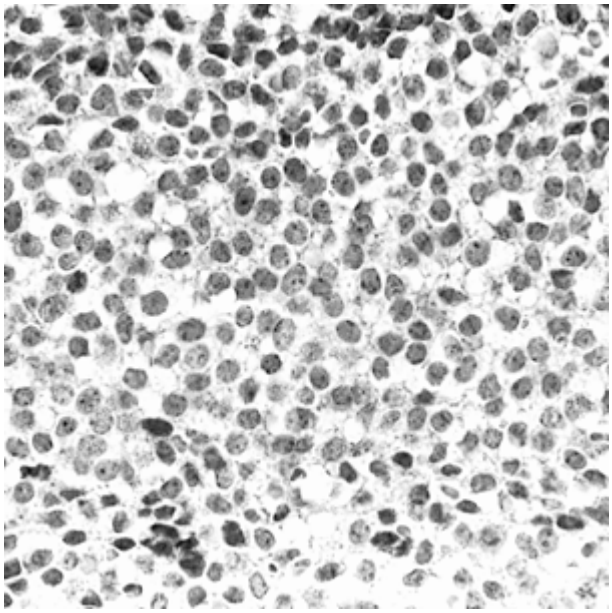

Fig. 1. Photomicrograph of the typical Ewing's sarcoma showing round tumor cells having nuclei that are fairly uniform and indistinct cytoplasmic border. The chromatin pattern is finely granular (H&E, ×400).

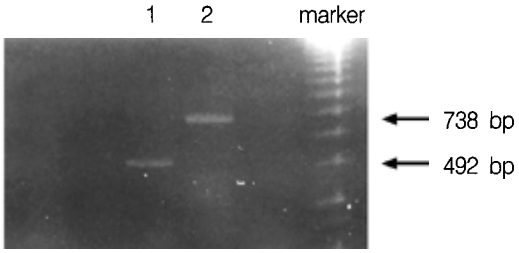

Fig. 2. Reverse transcription polymerase chain reaction analysis of formalin fixed paraffin embedded Ewing's sarcoma. Lane 1 and 2 represent positive results described in the text. Nested PCR products were separated on an agarose gel and stained with ethidium bromide.

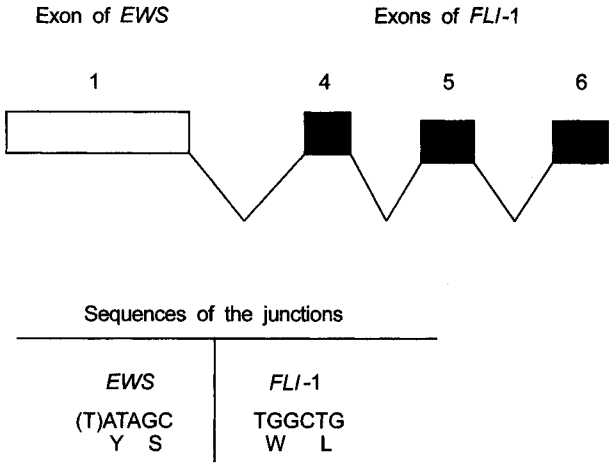

Fig. 3. EWS-FLI-1 mRNA junctions. Nucleotide sequence and deduced amino acid sequence of the junction are indicated.

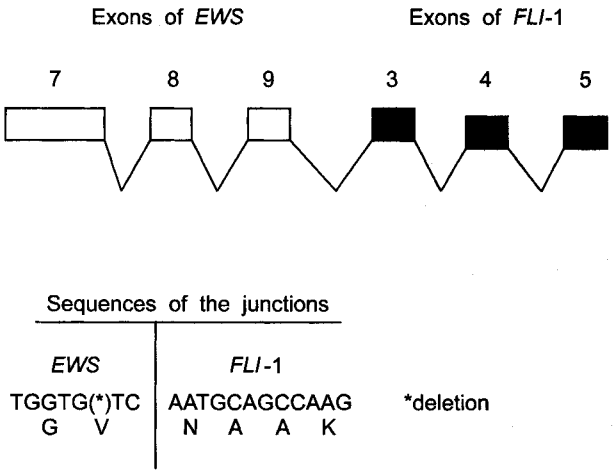

Fig. 4. EWS-FLI-1 mRNA junctions. Nucleotide sequence and deduced amino acid sequence of the junction are indicated.

staining positive granules which meant glycogen in the cytoplasm. In areas of necrosis, distinctive perivascular cuffs were noted. We did not include atypical Ewing's sarcoma composed of larger cells with more marked variation in nuclear size and shape, a clear or vesicular nucleus, and prominent nucleoli. The peripheral neuroectodermal tumors of bone showing Homer Wright rosettes and fibrillary intercellular background were also excluded in this study.

Formalin fixed paraffin embedded tumor tissue was used to isolate total RNA as described above. The purified RNA of most of the patients was nearly degraded.

Among the forty patients in this study, six cases of RNA were successfully isolated. To detect the *EWS/FLI*-1 fusion transcript, we performed a nested RT-PCR assay. Using the specific *EWS/FLI*-1 primers, positive amplification products (738 and 492 bp) were detected only after performing a nested PCR in two cases out of six patients (Fig. 2). Sequence analysis of the amplified band (492 bp) revealed an *EWS* exon 1/*FLI*-1 exon 4 fusion transcript (Fig. 3) and amplified band (738 bp) showed an *EWS* exon 9/*FLI*-1 exon 3 fusion transcript. In this patient, also noted were insertion of cytosine and point mutation of adenine to cytosine on the *FLI*-1 exon and deletion was noted at the location of adenine on the *EWS* exon (Fig. 4).

DISCUSSION

The typical Ewing's sarcoma is composed of small, round uniform cells. The nuclei are round and the nucleoli are inconspicuous. The nuclei have a rather smoky appearance. The cytoplasmic boundaries are indistinct. The differential diagnosis includes other small cell neoplasms, especially metastatic neuroblastoma and malignant lymphoma. Immunohistochemically, both Ewing's sarcoma and peripheral primitive neuroectoderal tumor stain positive for the MIC 2 gene product (3, 19). This O13 (HBA71;p30/32 MIC 2) is a cell membrane protein coded by a gene located on the short arms of the X and Y chromosomes (19). It is, however, not pathognomonic for this family of tumors, inasmuch as occasional expression of this marker has been documented in several unrelated types of tumor limits its reliability (3, 4, 6). In 1983, Aurias et al. discovered specific chromosomal translocation in Ewing's sarcoma to find a breakpoint has become one of the definite diagnostic tools (7). This translocation can be detected by conventional cytogenetics (7), Southern blot or Northern blot analysis (13), fluorescence in situ hybridization (FISH) on metaphase or interphase preparations (20-23) and RT-PCR (14-16, 24). In conventional cytogenetics and FISH, metaphase spreads require viable cells for short-term tissue culture. In Southern or Northern blot analysis, high-quality DNA or RNA is essential for detection of the specific chromosomal translocation. Until now, applied RT-PCR to detect the chromosomal translocation has also been restricted to fresh or snap-frozen tissue sample. For a pathologist, frozen tissue material is often not available, because tumor samples are formalin fixed and paraffin embedded as routine procedure. In this study, we used formalin fixed and paraffin embedded samples to detect chromosomal translocation. These new molecular diagnostic methods are used to support the difficult histological diagnosis of Ewing's sarcoma.

Currently, it is possible to isolate RNA from formalin fixed paraffin embedded tissue and to perform an RT-PCR assay (17, 25). Usually RNA extracted from the paraffin block presents more problems for molecular biology work such as RT-PCR because of the degradation connected with autologous RNase and with the fixation and paraffin embedding procedures. The method used for RNA extraction is basically consisted of a rehydration step of the deparaffinized section coupled to a proteolysis step with a high concentration of proteinase K in the presence of 1 M gunidinium thiocyanate. This allows an efficient RNA extraction without further degradation.

In this study, we could isolate six cases of good quality RNA out of forty Ewing's sarcoma patients. The rest of the cases showed degraded RNA. Although the RNA quality is poor and with our experience, the older the paraffin blocks, the worse the isolated RNA quality. Through this study, we could clearly demonstrate the fusion transcript *EWS/FLI*-1, generated by the t(11;22) balanced translocation in formalin fixed paraffin embedded tissue of Ewing's sarcoma. To increase the signal intensity and to confirm the specificity of the amplification product, we performed a nested PCR. This step of signal intensification leads to a crucial improvement in the subsequent detection of the t(11;22) translocation in formalin fixed paraffin embedded sample. In 1993, Sorensen et al. (15) used single PCR amplification to find translocation using RNA isolated from formalin fixed paraffin embedded tissue as template. However, their single PCR amplification was not sufficient to find chromosomal translocation in formalin fixed paraffin embedded tissue samples. We applied a nested PCR method and we could find the t(11;22)(q24;q12) chromosomal translocation. Therefore, a nested PCR method is essential to detect t(11;22)(q24;q12) in formalin fixed paraffin embedded tissue samples.

One limitation of our protocol must be noted. In cases where tissue material has been decalcified with nitric acid, no amplification product was visible. Furthermore, quality control of the isolated RNA is essential, and only material in which a housekeeping gene, like b-actin, is possible to be amplified should be used for the t(11;22) translocation analysis.

In conclusion, with the method described in this study, the pathologist is now able to apply new molecular biology techniques such as RT-PCR for the detection of t(11;22) chromosomal translocation on paraffin embedded samples to make a definite diagnosis of Ewing's sarcoma.

Acknowledgments

This work was supported in part by the 1997 Kyung Hee University Hospital Research Fund.

REFERENCES

1. Fechner RE, Mills SE. *Atlas of tumor pathology, tumors of the bones and joints. AFIP third series, Bethesda, 1993: 187.*
2. Kissane JM, Askin FB, Foulkes M, Stratton LB, Shirley SF. *Ewing's sarcoma of bone: clinicopathologic aspects of 303 cases from the intergroup Ewing's sarcoma study. Hum Pathol 1983; 14: 773-9.*
3. Ambros IM, Ambros PF, Strehl S, Kovar H, Gadner H, Salzer-Kuntschik M. *MIC2 is a specific marker for Ewin's sarcoma and peripheral primitive neuroectodermal tumors. Cancer 1991; 67: 1886-93.*

4. Fellinger EJ, Garin-Chesa P, Triche TJ, Huvos AG, Rettig WJ. *Immunohistochemical analysis of Ewing's sarcoma cell surface antigen p30/32^{MIC2}. Am J Pathol 1991; 139: 317-25.*
5. Hamilton G, Fellinger EJ, Schratter I, Fritsch A. *Characterization of a human endocrine tissue and tumor-associated Ewing's sarcoma antigen. Caner Res 1988; 48: 6127-31.*
6. Kovar H, Dworzak M, Strehl S, Schnell E, Ambros IM, Ambros PF, Gadner H. *Overexpression of the pseudoautosomal gene MIC2 in Ewing's sarcoma and peripheral primitive neuroectodermal tumor. Oncogne 1990; 5: 1067-70.*
7. Aurias A, Rimbaut C, Buffe D, Dubousset J, Mazabraud A. *Chromosomal translocations in Ewing's sarcoma. N Engl J Med 1983; 309: 496-7.*
8. Turc-Carel C, Philip I, Berger MP, Philip T, Lenoir GM. *Chromosomal translocations in Ewing's sarcoma. N Engl J Med 1983; 309: 497-8.*
9. Delattre O, Zucman J, Melot T, Garau XS, Zucer JM, Lenoir GM, Ambros PF, Sheer D, Phil D, Turc-Carel C, Triche TJ, Aurias A, Thomas G. *The Ewing family of tumor-a subgroup of small round cell tumors defined by specific chimeric transcripts. N Engl J Med 1994; 331: 294-9.*
10. Ladanyi M, Lewis R, Garin-Chesa P, Rettig WJ, Huvos AG, Healey JH, Thanwar SC. *EWS rearrangement in Ewing's sarcoma and peripheral neuroectodermal tumor. Molecular detection and correlation with cytogenetic analysis and MIC2 expression. Diagn Mol Pathol 1993; 2: 141-6.*
11. Turc-Carel C, Aurias A, Mugneret F, Lizard S, Sidaner I, Vol C, Thiery JP, Olschwang S, Philip I, Berger MP. *Chromosomes in Ewing's sarcoma. I. An evaluation of 85 cases of remarkable consistency of t(11;22)(q24;q12). Cancer Genet Cytogenet 1988; 32: 229-38.*
12. Noguera R, Navarro S, Triche TJ. *Translocation (11;22) in small cell osteosarcoma. Cancer Genet Cytogenet 1990; 45: 121-4.*
13. Zucman J, Delattre O, Desmaze C, Plougastel B, Joubert I, Melot T, Peter M, De Jong P, Rouleau G, Aurias A, Thomas G. *Cloning and characterization of the Ewing's sarcoma peripheral neuroepithelioma t(11;22) translocation breakpoints. Genes Chromosom Cancer 1992; 5: 271-7.*
14. Dellatre O, Zucman J, Plougastel B, Desmaze C, Melot T, Peter M, Ovar H, Joubert I, De Jong P, Rouleau G, Aurias A, Thomas G. *Gene fusion with the ETS DNA-binding domain caused by chromosome translocation in human tumors. Nature 1992; 35: 162-5.*
15. Sorensen PH, Liu XF, Delattre O, Rowland JM, Biggs CA, Thomas G, Triche TJ. *Reverse transcriptase PCR amplification of EWS/FLI-1 fusion transcripts as a diagnostic test for peripheral primitive neuroectodermal tumors of childhood. Diagn Mol Pathol 1993; 2: 147-57.*
16. Zucman J, Melot T, Desmaze C, Ghydael J, Plougastel B, Peter M, Sucker JM, Triche TJ, Sheer D, Turc-Carel C. *Combinatorial generation of variable fusion proteins in the Ewing family of tumors. EMBO J 1993; 12: 4481-7.*
17. Stanta G, Schneider C. *RNA extracted from paraffin-embedded human tissues is amendable to analysis by PCR amplification. Biotechniques 1991; 11: 304-8.*
18. Sanger F, Nicklen S, Coulson AR. *DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74: 5463-7.*
19. Weidner N, Tjoe T. *Immunohistochemical profile of monoclonal antibody 013: antibody that recognizes glycoprotein p30/32^{MIC2} and is useful in diagnosing Ewing's sarcoma and peripheral neuroepithelioma. Am J Surg Pathol 1994; 18: 486-94.*
20. Desmaze C, Zucman J, Delattre O, Thomas G, Aurias A. *Unicolor and bicolor in situ hybridization in the diagnosis of peripheral neuroepithelioma and related tumors. Genes Chromosom Cancer 1992; 5: 30-4.*
21. Desmaze C, Zucman J, Delattre O, Melot T, Thomas G, Aurias A. *Interphase molecular cytogenetics of Ewing's sarcoma and peripheral neuroepithelioma t(11 ; 22) with flanking and overlapping cosmid probes. Cancer Genet Cytogenet 1994; 74: 13-8.*
22. Giovannini M, Selleri L, Biegel JA, Scotland K, Emanuel BS, Evans GA. *Interphase cytogenetics for the detection of the t(11;22)(q24;q12) in small round cell tumors. J Clin Invest 1992; 90: 1911-8.*
23. Taylor C, Patel K, Jones T, Kiely F, Stavola BL, Sheer D. *Diagnosis of Ewing's sarcoma and peripheral neuroectodermal tumor based on the detection of t(11;22) using fluorescence in situ hybridization. Br J Cancer 19993; 67: 128-33.*
24. Downing JR, Head DR, Parham DM, Douglass ED, Hulshof MG, Link MP, Motroni TA, Grier HE, Curcio-Brint AM, Shapiro DN. *Detection of the t(11;22)(q24;q12) translocation of Ewing's sarcoma and peripheral neuroectodermal tumor by reverse transcription polymerase chain reaction. Am J Pathol 1993; 143: 1294-300.*
25. Finke J, Fritzen R, Ternes P, Lange W, DolkenG. *An improved strategy and a useful housekeeping gene for RNA analysis from formalin fixed paraffin embedded tissues by PCR. Biotechniques 1993; 14: 448-53.*