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Expression in *E. coli* and purification of a chimeric p22-NS3 recombinant antigen of Hepatitis C Virus (HCV)

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A recombinant antigen (p22-NS3), possessing putative HCV nucleocapsid protein (p22) and non-structural protein 3 (NS3) epitopes, was heavily expressed in *E. coli* and purified. The p22-NS3 purified recombinant antigen strongly reacts with sera containing human antibodies directed against p22 and NS3 providing a starting point for the design of an HCV single all-encompassing antigen for a blood screening assay.

Hepatitis C virus; Antigen; Fusion protein; Purification, Antibody

1. INTRODUCTION

Non-A non-B hepatitis (NANBH) is a transmittable disease for which the etiological agents are distinct from hepatotropic viruses such as hepatitis A, B and delta [1]. The etiological agent of NANBH was identified by Choo and coworkers in 1989 [2–5] and denominated hepatitis C virus (HCV). The viral genome is a positive stranded RNA of about 9.4 kb [2] encoding a putative polyprotein of 3010–3011 amino acids [6–10]. The HCV genome codes for three structural proteins (putative nucleocapsid protein, putative matrix, envelope) and six non-structural proteins (NS1, NS2, NS3, NS4a, NS4b and NS5) [7,11].

For diagnosis of HCV infection, structural and non-structural regions of HCV-encoded polyprotein have been expressed in various heterologous systems [12–14]. The recombinant proteins were used either individually or combined to capture anti-HCV antibodies [12,15,16]. However, immunological studies have shown that the most important epitopes lie within the putative nucleocapsid protein (p22) [13,17] and in the non-structural protein 3 (NS3) and 4 (NS4) [18]. In this paper we evaluate the usefulness of a chimeric protein containing epitopes of both p22 and NS3 for the design of a HCV single all-encompassing antigen for a blood screening assay. We have expressed in *E. coli* a chimeric protein made of amino acids 1–105 of p22 and amino acids 1186–1364 of NS3 and we have tested its immunoreactivity against anti-HCV positive sera. The purified chimeric protein (p22-NS3) was shown to bind to sera from HCV infected patients showing anti-p22 and anti-NS3 immunoreactivities.

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Abbreviations: HCV, hepatitis C virus; anti-HCV, anti-HCV antibodies; NS, non structural; p22, nucleocapsid protein; p22-NS3, recombinant chimeric antigen; NANBH, non-A non-B hepatitis; Anti-p22, anti-nucleocapsid antibodies; Anti-NS3, anti-NS3 antibodies; CH_6ClN_3, guanidinium chloride; rec, baculovirus recombination sequence; Pl, lambda promoter.

2. MATERIALS AND METHODS

2.1. *Materials*

Culture media, salts, and solvents were purchased from Sigma (USA); gel-filtration resins were purchased from Pharmacia (Sweden); oligonucleotides were synthesized using a Beckman Synthesizer (USA); panels of certified HCV positive and negative sera were purchased from BBI (USA); plasmid pVL1393 was purchased from Stratagene (USA), plasmid pGEX-3X was purchased from Promega (USA); Anti-HCV antibody detection kits were supplied by SORIN Biomedica (Italy).

2.2. *Cloning of p22-NS3 in E coli*

Partial HCV p22 gene (nucleotide position −21 to 491) and partial NS3 gene (nucleotide position 3549 to 4092) were obtained from lambda clones of HCV genome fragments (F. Bonelli, unpublished results) using polymerase chain reaction amplification (PCR) [20]. The cloning strategy is described in Fig. 1.

2.3 *Expression of p22-NS3 in E coli*

Bacterial culture (RR1) [21] transformed with pEV22-NS3 was grown at 30°C in LB medium [22] (500 ml) supplemented with 50 mg/l ampicillin When the cells density reached an A_{590} = 0 5 the culture was transferred to 42°C. After 4 h induction, cells were collected by centrifugation.

2.4. *Cellular lysis*

E. coli cells (7.6 g), strain RR1, transformed with pEV22-NS3, were suspended in 19 ml buffer A (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA). The suspension was sonicated (3 × 100 W burst, 5°C) and then clarified by centrifugation (20 min, 10,000 × *g*, 10°C). Cellu-

lar debris were subsequently extracted with 12 ml buffer B (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% Triton X-100) (60 min, 4°C). The solution was clarified by centrifugation as described above. The pellet was dissolved in buffer C (7 M CH_6ClN_3, 50 mM sodium acetate pH 5.0, 10 mM β-mercaptoethanol), incubated for 30 min at 60°C, 5 min at 95°C and then the solution was clarified by centrifugation. An aliquot of the clarified solution (0.1 ml) was dialized against buffer D (8 M urea, 50 mM HEPES pH 7.0, 10 mM β-mercaptoethanol). 1 μl of the dialized solution was loaded on a 12.5% acrylamide SDS PAGE (Fig. 2, lane 2).

2.5. *Gel-filtration chromatography*

The clear supernatant was loaded onto a Sephacryl S300 HR gel-filtration column (2.5 cm × 100 cm) equilibrated in buffer C (flow rate = 2 ml/min, detector 280 nm). The eluted material was collected in 5 ml fractions The peak fractions, containing the higher level of p22-NS3 immunological reactivity (hatched peak, Fig. 3A), were pooled. The immunoreactive peak was dialized against buffer D 1 μl of the dialized pool was loaded on a 12 5% acrylamide SDS-PAGE (Fig. 2, lane 3).

2.6. *Ion exchange chromatography*

The gel filtration pool, after dialysis, was loaded onto a CM-Sepharose FF column (15 mm × 40 mm) equilibrated in buffer D (flow rate = 2 ml/min, detector 280 nm). The unbound material was washed out with buffer D until the base line returned to zero. The absorbed proteins are eluted with 0.5 M NaCl in buffer D (flow rate 2 ml/min). The peak fractions, containing the higher level of p22-NS3 immunological reactivity (hatched peak, Fig. 3B), were pooled. 1 μl of the dialized pool was loaded on a 12.5% acrylamide SDS PAGE (Fig. 2, lane 4).

2.7. *p22-NS3 refolding*

The p22-NS3 recombinant polypeptide was refolded in vitro as described by J.L. Cleland [23]. Purified p22-NS3 (30 μM, 3 ml), in presence of PEG 3350 with a PEG to p22-NS3 molar ratio of 10 to 1, was dialyzed against 500 ml 8 M urea in buffer E (100 mM HEPES pH 6.0, 0.5 M NaCl) using a 1 kDa cut-off dialysis tube, under nitrogen atmosphere at 4°C. The urea concentration in buffer E was reduced to zero by a step of 2 M every 2 hours. Then the p22-NS3 was extensively dialyzed against buffer E. At the end of the refolding procedure the recombinant polypeptide p22-NS3 was soluble in buffer E (100 mM HEPES pH 6 0, 0.5 M NaCl).

2.8. *Western blot and ELISA immunoassays*

Western blot analysis was done on purified p22-NS3 recombinant protein, with sera from HCV infected patients showing anti-NS3 and anti-p22 immunoreactivities (data not shown), using a modified Towbin's protocol [24] p22-NS3 immunoreactivity detection in column eluates (data not shown) was performed using Western Blot analysis, with anti-p22 IgG purified from a pool of HCV infected human sera. ELISA assays (Fig. 4) were performed as previously described [25]

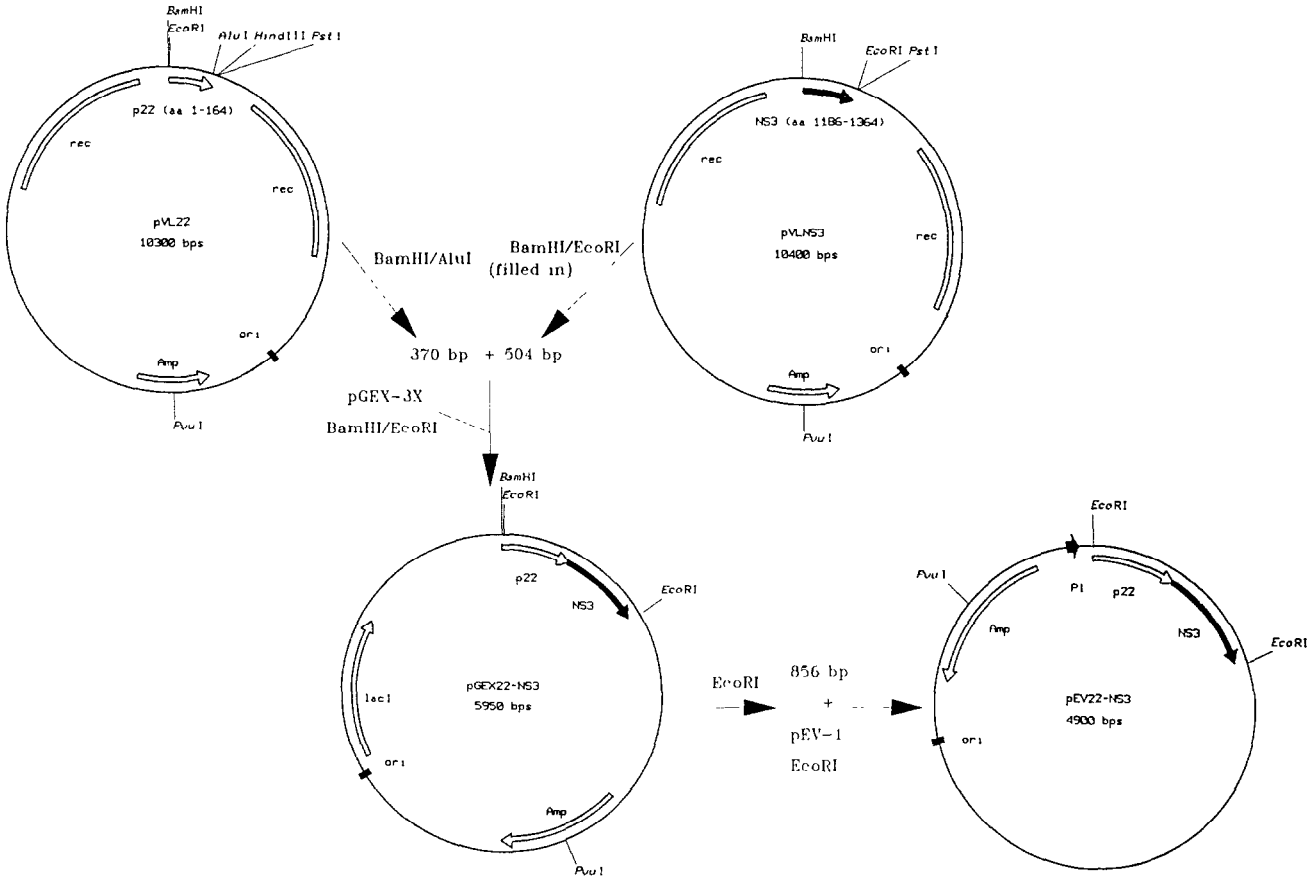

Fig. 1 The p22 and NS3 partial gene fragments were cloned in pVL1393 (pVL22, pVLNS3) (R. Calogero, personal communication). The gene fragment *Bam*HI/*Alu*I from pVL22, encoding amino acids 1 to 105 of p22 gene, was ligated with fragment *Bam*HI (filled in)/*Eco*RI from pVLNS3, encoding amino acids 1186–1364 of NS3 gene. The chimeric gene was cloned in pGEX-3X cut *Bam*HI/*Eco*RI (pGEX22-NS3), excised by *Eco*RI digestion and cloned in pEV1 [20] cut *Eco*RI (pEV22-NS3).

3. RESULTS AND DISCUSSION

We have cloned (Fig. 1) and expressed in *E. coli* a recombinant chimeric p22-NS3 antigen. Nucleotide sequence analysis (data not shown) of the partially cloned p22 and NS3 genes has shown homology higher than 97%, at the amino acid level, with published HCV p22 and NS3 [6].

The purified recombinant p22-NS3 is a fusion protein consisting of 3 amino acids (M-N-E) of pEV1 linker [20], 9 amino acids (F-R-S-R-R-P-C-I-M) of pVL1393 polylinker, HCV nucleocapsid protein sequence truncated at amino acid 105 and the central region of NS3 protein from amino acid 1186 to 1364. Recombinant antigen has a molecular weight of 33 kD (Fig. 2, lane 4). The sequencing of the first 20 amino acids at the NH2-terminus confirmed the sequence predicted from the DNA sequencing. The antigen accumulates inside the *E. coli* cells in inclusion bodies and represents about 2% of the total cell proteins (data not shown). We have purified the antigen using a two-steps procedure involving gel-filtration and cationic exchange chromatography (Fig. 3A,B). The immunological behavior of the recombinant p22-NS3 after gel-filtration was quite poor. We have observed false positive reactivities using the gel-filtration purified p22-NS3 (data not shown). However, following cationic exchange chromatography the recombinant antigen has shown a specific binding to sera having anti-p22 and anti-NS3 immunoreactivities (Fig. 4). Moreover the protein content of the unbound material, eluted from the ionic exchange column, was less than 1/100 of the purified recombinant antigen. At the end of the purification procedure the protein was over 95% pure as seen by Coomassie staining (Fig. 2, lane 4) and the final yields were of the order 3 mg per liter culture. Recombinant antigen after purification

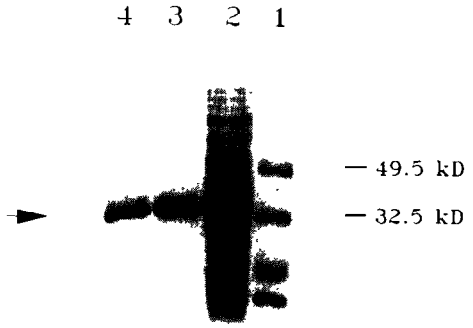

Fig. 2. In the SDS PAGE analysis recombinant p22-NS3 is shown by an arrow. Lane 1, Bio-Rad prestained molecular weight markers; lane 2, *E. coli* debris dissoved in buffer C; Lane 3, peak fraction pool of S300 HR gel-filtration, associated with p22-NS3 immunoreactivity; lane 4, peak fraction pool of ionic exchange chromatography, associated with p22-NS3 immuno-reactivity.

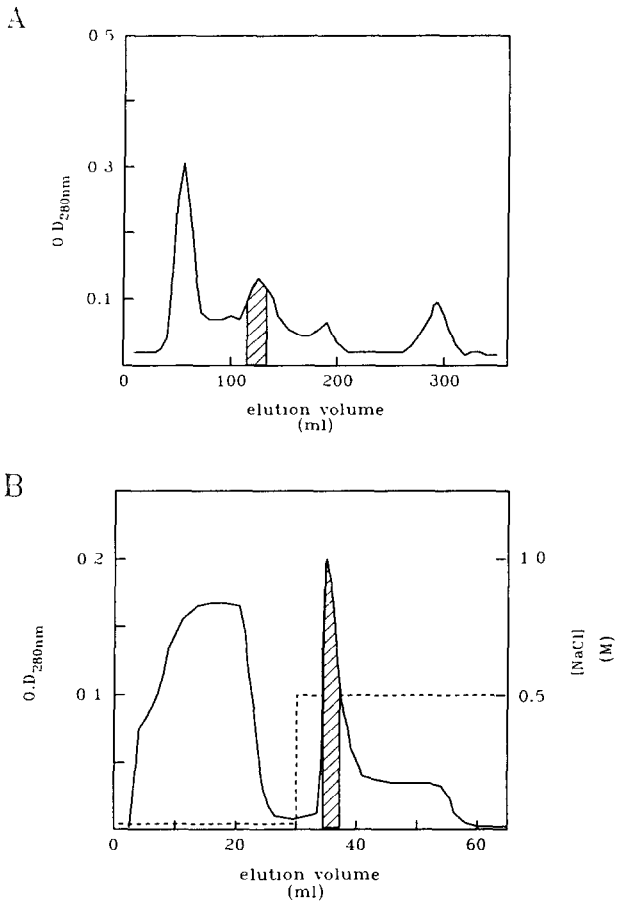

Fig. 3. (A) Elution profile of gel-filtration chromatography (see section 2). (B) Elution profile of ionic exchange chromatography (see section 2).

was soluble only in strong denaturants such as urea and guanidinium chloride. We have used the refolding procedure proposed by J.L. Cleland [23] to obtain a recombinant p22-NS3 soluble in non-denaturant solutions. The immunoreactivity of p22-NS3 was compared, in indirect ELISA assays, with respect to recombinant p22 (R. Calogero personal communication). The chimeric p22-NS3 protein has shown to react not only with sera having anti-p22 immunoreactivity, but also with patient sera containing human antibodies directed against NS3 (Fig. 4, closed bars: sera 8, 10 and, as border line value, serum 20). On the other hand recombinant p22 can interact only with sera having anti-p22 immunoreactivity (Fig. 4, open bars).

The level of purity and the yield of protein, which we achieved, will allow the easy coupling of p22-NS3 with enzymes or fluorescent compounds for development of antigen labeled sandwich immunoassays [26].

It was shown that anti-p22, anti-NS3 and anti-NS4 immunoreactivities are present in different combinations in the early stage of HCV infection [18]. However, an HCV single all-encompassing antigen for blood

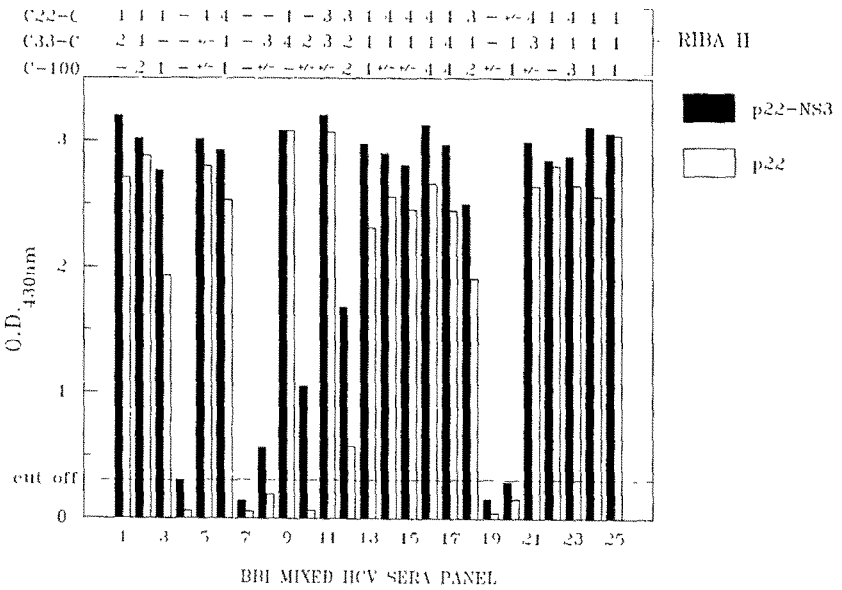

Fig. 4. Immunoreactivity of recombinant p22-NS3 as compare to recombinant p22 antigen was evaluated analyzing their binding to anti-HCV antibodies, using a certified anti-HCV positive and negative sera panel (BBI, USA). Closed bars show the immunoreactivities identified with chimeric p22-NS3. Open bars show the immunoreactivities identified with recombinant p22. On the top of the figure the values for p22 (C22-c), NS3 (C33-c) and NS4 (C-100) immunoreactivities are shown. These data were obtained by manufacturer using a second generation RIBA assay for detection of anti-HCV antibodies (Ortho Diagnostics, USA). Values 4 to 1 indicate clear positive reactivities, +/− and −, respectively, indicate reactivities near cut off value and absence of reactivity.

screening was not yet developed. The p22-NS3 recombinant chimeric antigen provides a starting point for the assembly of a multi-HCV-epitopes polypeptide.

REFERENCES

[1] Alter, H.J., in: Current Perspectives in Hepatology (Seeff, L.B. and Lewis, J H., Eds.) pp. 83–97, Plenum, New York
[2] Choo, Q-L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W and Houghton, M. (1989) Science 244, 359–362.
[3] Kuo, G., Choo, Q.-L., Alter, H J., Gitnick, G L., Redeker A.G., Purcell, R.H., Miyamura, T., Dienstag, J L., Alter, M J., Stevens C E., Tegtmeier, G.E., Bonino, F., Colombo, M., Lee, W.S., Kuo, C., Berger K., Shuster, J.R., Overby, L.R., Bradley, D.W. and Houghton, M. (1989) Science 244, 362–364.
[4] Alter, H J., Purcell, R H., Shih, J W., Melpolder, J.C., Houghton, M., Choo, Q.-L and Kuo, G. (1989) Engl. J Med. 321, 1494–1500.
[5] Miyamura, T., Saito, I., Katayama, T., Kikuchi, S., Tateda, A., Houghton, M., Choo, Q.-L and Kuo, G (1990) Proc Natl. Acad. Sci. USA 87, 983–987.
[6] Houghton, M., Choo, Q.-L. and Kuo, G., European Patent Application 90302866.0 and Publication 0 388 232 (1990).
[7] Houghton, M., Richman, K., Han, J., Berger, K., Lee, C., Dong, C., Overby, L., Weiner, A., Bradley, D., Kuo, G and Choo, Q.-L. (1991) in: Viral hepatitis and liver disease (Hollinger, F B., Lemon, S.M and Margolis, H.S. Eds.) pp. 328–333, William and Wilkins, Baltimore.
[8] Kato, N., Hijikata, M., Ootsuyama, Y., Nagakawa, M., Ohkoshi, S., Sugimura, T. and Shimotohno, K. (1990) Proc. Natl Acad Sci. USA 87, 9524–9528.
[9] Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I. and Okayama, H. (1991) J. Virol 65, 1105–1113.
[10] Takeuchi, K., Kubo, Y., Boonmar, S., Watanabe, Y., Katayama, T., Choo, Q.-L., Kuo, G., Houghton, M., Saito, I. and Miyamura, T. (1990) Nucleic Acids Res 18, 4626.
[11] Choo, Q.-L., Richman, K.H., Han, J H., Berger, K., lee, C., Dong, C., Gallegos, C., Colt, D., Medina-Selby, A., Barr, P.J., Weiner A J., Bradley, D.W., Kuo, G. and Houghton, M. (1991) Proc Natl. Acad. Sci USA 88, 2451–2455.
[12] Chiba, J., Ohba, H., Matsuura, Y., Watanabe, Y., Katayama, T., Kikuchi, S., Saito, I. and Miyamura, T. (1991) Proc. Natl. Acad Sci. USA 88, 4641–4645.
[13] Harada, S., Watanabe, Y, Takeuchi, K., Suzuki, T., Katayama, T., Takebe, Y., Saito, I. and Miyamura, T (1991) J Virol. 65, 3015–3021.
[14] Chien, D Y., Choo, Q-L., Tabrizi, A., Kuo, C., McFarland, J., Berger, K., Lee, C., Shuster, J.R., Nguyen, T., Moyer, D.L., Tong, M., Furuta, S., Omata, M., Tegtmeier, G., Alter, H., Schiff, E., Jeffers, L., Houghton, M and Kuo, G. (1992) Proc Natl. Acad. Sci. USA 89, 10011–10015.
[15] Muraiso, K., Hijikata, M., Kato, N., Shimotohno, K., Okazaki, N., Ohkoshi, S., Uura, M., Kaneko, S., Kobayashi, K. and Omata, M. (1991) Jpn. J. Cancer Res. 82, 879–882
[16] Van der, Poel, C.L., Cuypers, H.T., Reesink, H W., Weiner, A J., Quan, S., Di, Nello, R., Van, Boven, J.J and Winkel, I (1991) Lancet 337, 317–319.
[17] Nasoff, M S., Zebedee, S.L., Inchauspe, G and Prince, A.M. (1991) Proc. Natl. Acad Sci USA 88, 5462–5466
[18] Beach, M.J., Meeks, E.L., Mimms, L.T., Vallari, D., DuCharme L., Spelbring, J., Taskar, S., Scheicher, J.B., Krawczynski, K. and Bradley, D.W. (1992) J Med Virol. 36, 226–237.
[19] Scharf, S.J., Horn, G.T and Erlich, H.A. (1986) Science 233, 1076–1078.
[20] Crowl, R., Seamans, C., Lomedico, P and McAndrew, S. (1985) Gene 38, 31–38
[21] Bolivar, F. and Backman, K. (1979) Methods Enzymol. 68, 482–492
[22] Maniatis, T., Fritsch, E.F and Sambrook, J. (1982) in. Molecular

Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[23] Cleland, J.L., Builder, S.E., Swartz, J.R., Winkler, M., Chang, J.Y and Wang, D.I.(1992) Bio/Technology 10, 1013–1019.
[24] Talbot, P.V., Knobler, R.L and Buchmeier, M. (1984) J. Immunol. Methods 73, 177–188
[25] Osborne, S., Cecconato, E., Griva, S., Calogero, R., Rosa, C. and Bonelli, F. (1993) Prot. Eng. 6, 68.
[26] Siitari, H., Turunen, P., Schrimsher, J. and Nunn, M. (1990) J. Clin. Microbiol. 28, 2022–2029.