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A Novel Protein Refolding Protocol for Solubilization and Purification of the Catalytic Fragment of Recombinant MMuLV Reverse Transcriptase Overexpressed in Escherichia Coli

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Abstract— Reverse transcriptase (RT) from Moloney murine leukemia virus (MMuLV) is an RNA-dependent DNA polymerase that uses single-stranded RNA, DNA, or an RNA-DNA hybrid to synthesize a cDNA strand. The purification methods available for this enzyme are laborious and not cost-effective. We present a novel and efficient protein refolding protocol for solubilization and purification of the catalytic fragment of MMuLV-RT. Our protocol is based on the denaturation of the RT enriched inclusion bodies with an alkaline solution of 8 M urea, followed by refolding in a refolding buffer and single buffer-exchange steps via dialysis. The protocol proved effective for us to obtain a large amount of relatively pure and rightly folded RT protein that exhibited potent reverse transcriptase activity on HIV and HCV viral RNA's comparable to commercially available MMuLV RT enzyme preparation. The yield of ~23 mg of purified RT/litre of *E. coli* culture is the highest yield reported to date and the purification process presented in this article is scalable with high recovery rate.

Keywords— Mmulv Reverse Transcriptase; Protein Refolding; Ni-NTA Chromatography; HCV Diagnosis; HIV Diagnosis.

I. INTRODUCTION

Moloney murine leukemia virus (MMuLV) reverse transcriptase (RT) [EC 2.7.7.49] is widely used in cDNA synthesis for RT-polymerase chain reaction (PCR). Due to its high catalytic activity and fidelity, RT is also considered in microarray analysis using Rapid Amplification of cDNA Ends (RACE) and Transcription Mediated Amplification (TMA) (Kievits et al., 1991). This RNA-dependent DNA polymerase, i.e., RT, uses single-stranded RNA, DNA, or an RNA-DNA hybrid for the synthesis of a cDNA strand (Roth et al., 1985).

RTs exhibit two catalytic activities: a DNA polymerase activity and an associated RNase activity. The DNA polymerase activity at the N terminal extends the 3' end of a primer and copies either RNA or DNA templates as required to form the first and second strands of the viral DNA. Comparatively, the RNase activity, termed RNase H, degrades RNA at the C terminus only when it is in the form of an RNA-DNA hybrid duplex since the single-stranded RNA and RNA-RNA duplexes are resistant to degradation (Tanese and Goff, 1988).

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MMuLV RT is a single 75 kDa monomer inhibited by polyamines, phosphate, pyrophosphates, and lithium chloride (Gerard and D'Alessio, 1993). It has a much lower RNase H activity than AMV RT and hence yields high amounts of full-length cDNA (Sambrook and Russell, 2001), although the avain enzyme is known to show better thermal stability than the MMuLV.

The full-length molecule of MMLV RT showed RNA- and DNA-dependent DNA polymerase and RNase H activities. The DNA polymerase reaction is active in the fingers/palm/thumb domain while the RNase H reaction is active in the RNase H domain. MMuLV RT is not thermostable since it loses 50% of its initial reverse transcription activity after exposure at 44 °C for 10 min (Yasukawa et al., 2008). However, this can be improved by eliminating RNase H activity (Kotewicz et al., 1985; Gerard et al., 2002; Mizuno et al., 2010). The mutations were multiple and mainly focused on eliminating the RNaseH activity and stabilizing the reverse transcription activity (Konishi et al., 2014). These efforts made the RT molecule retain 70% of its activity after thermal incubation at 50 °C for 10 min. RNase H can degrade templates when longer initial incubation times are required for synthesizing long cDNAs; hence, an RT molecule without RNase H activity seemed beneficial for this application. Since the two domains can be separately expressed in *Escherichia coli* and still retain their respective activities[3], M-MLV RT (H+) has been successfully used for analytical and preparative cDNA applications. In addition, RTs without RNase H activity provide another option to

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prepare long cDNAs and libraries containing a high percentage of full-length cDNA.

Detection of viral RNA by PCR requires the prior reverse transcription of viral RNA. Sellner et al. (1992) reported that increasing the ratio of Taq to RT and addition of non-homologous RNA improves the sensitivity of the RT activity. In addition, the effect is not limited to AMV RT, MMLV-RT also affects Taq activity.

Although recombinant full-length MMLV-RT has been produced in *E. coli* (Tanese et al., 1985; Baranauskas et al., 2012; Chen et al., 2009) and cloning and successful purification of both the catalytic and the RNaseH domains are active separately in *E. coli* (Tanese and Goff, 1988), all the protocols used for purification conditions are laborious requiring multiple steps, which compelled us to develop a protocol for easy purification of the RT protein. Moreover, since there is only a single report on the cloning, expression and purification of the catalytic fragment of the MMLV RT as a soluble protein with multiple steps of purification (Sun et al., 1998), we envisaged that having a universal refolding purification protocol from inclusion bodies would be useful for researchers worldwide.

Since MMLV RT without the RNaseH domain has been reported to exhibit higher thermostability (4-fold longer half-life at 50 °C) compared to wild-type MMLV RT (Gerard et al., 2002), we opted for cloning and expressing only the catalytic fragment of the MMuLV-RT. Hence, this work assumes critical importance.

There are numerous reports on successful expression of full-length RT of MMuLV. However, M-MLV RT RNase H– point mutant is much more thermostable, due to its single amino acid substitution that reduces RNase H activity but preserves full DNA polymerase activity. It can be used at temperatures of up to 55°C (Gerard et al., 2002), compared to wild-type M-MLV RT with optimal reaction temperature of 37 °C, making it suitable for RNAs with strong secondary structure.

One major issue in the mass production of MMLV RT in prokaryotic system is the limited solubility of the full-length 75 kDa enzyme, which could be due to requirement of successful refolding of the multiple functional domains of the MMuLV RT namely the fingers, palm, thumb, connection, and RNase H domains (Georgiadis et al., 1995; Das and Georgiadis, 2004; Lim et al 2006; Cote et al., 2008).

In this article, we demonstrate the expression of the catalytic fragment of the MMuLV -RT (henceforth, designated as C-RT) as an N terminal histidine-tag protein. The purified MMLV C-RT was not further processed for histidine-tag removal since it was small and less disruptive to the properties of the expressed proteins.

II. MATERIALS AND METHODS

A. Materials

The synthetic gene catalytic fragment of the MMuLV RT gene (C-RT) was synthesized from GenScript, USA. Reduced and oxidized glutathione, imidazole, DTT were procured from Amresco, USA while the Ni-NTA agarose was from Qiagen, USA. Igepal 630 was from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade, unless mentioned otherwise.

B. C-RT cloning and overexpression

The full-length MMuLV RT contains 671 amino acid residues (Cote al., 2008). The C-RT gene from 80 to 274 amino acids was cloned as Nde1/HindIII fragment in pET26b vector (Novagen, San Diego, USA) with 6 histidine residues at the N terminus along with an enterokinase cleavage site (DDDDK). The resultant size of the expected protein was 23 kDa. The recombinants of pET26b-C-RT were selected by PCR and restriction digestion, and the plasmids from positive clones were introduced into ER2566 (NEB, USA) competent cells. The transformants were grown in plain Luria Broth with Kanamycin at 10 µg/ml and later induced with 1 mM IPTG for 4 h at 37 °C. The induced pellet was re-suspended in 5 ml of 50 Mm Tris, pH 8.5 with 100 µl of (100 mg/ml) lysozyme and sonicated at 40 pulse and 50 watts power. The sonicated material was centrifuged at 10,000 rpm for 10 min, and the supernatant and the pellet fractions were separated for sub-cellular localization of the expressed protein.

C. Protein denaturation, refolding and purification

The induced pellet from 250 ml of the induced culture was subjected to three washes to remove all the contamination proteins from the inclusion body preparation containing the protein of interest. The first wash (wash 1) included 0.1% Triton X-100 in 50 mM Tris.Cl, pH 8.5 while wash 2 was made with 1M sodium chloride in 50 mM Tris.Cl, pH 8.5. The wash 3 solution contained 2M urea in 50 mM Tris.Cl, pH 8.5. The induced sonicated pellet was washed with 20 ml of each wash solution, and the washing was done in a rocker at RT for 15 min. The pellet obtained after the third wash was used for denaturation and purification studies.

The washed inclusion bodies containing the RT protein were re-suspended in 5 ml of 8M urea, pH 12.0. The contents were allowed to mix in a rocker for 15 min. The solution was clarified by spinning at 13000 rpm for 10 min to remove any undissolved debris, and this solution was added drop-wise to the refolding buffer (1 x PBS, pH 7.5; 10% glycerol, 0.3 and 3 mM oxidized and reduced glutathione respectively, 0.5% Triton X 100 and 0.4 M sucrose). The refolding was allowed overnight under stirring conditions in cold.

The refolded protein was spun after 16 h at 10,000 rpm for 30 min, and the clear refolded protein was bound to Ni-NTA column equilibrated with 1 x PBS, pH 7.4 with 100 mM NaCl.

The refolded material was bound to Ni-NTA in cold, and the bound proteins were eluted with imidazole step gradients in cold. Fractions containing the protein of interest were then dialyzed against 100 volumes of pre-storage buffer (20 mM Tris-HCl, pH 7.5, 0.1M NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% Igepal CA-630, 10% glycerol). The next day, the pH of the dialysate was adjusted to 6.0 with MES buffer along with 40% glycerol, and the protein in storage buffer was stored at -20 °C until use.

Silver staining was carried out by the method of Nesterenko, et al. (1994). Protein concentrations were determined by Bradford's method (Bradford, 1976) to quantify the total yield of C-RT per liter of induced culture under the described experimental conditions.

D. Processing of clinical samples

HIV and HCV ELISA positive plasma samples were obtained from Dr. C. Nagaraj, Kuppam Hospital, India. All the samples were handled with adequate precautions in BSL-II facility of Cancyte Technologies Pvt. Ltd, Bangalore, India. IVD certified NucleospinDx virus kit (Macherey-Nagel, GmbH) was used to isolate HIV and HCV RNA from clinical samples as per manufacturer's instructions.

E. Determination of the MMuLV C-RT enzyme activity

RTs use RNA as a template and specific primers complementary to the either ends of the RNA's direct the synthesis of the first strand cDNA, which can be used directly as a template for PCR. This RT-PCR allows the detection of low abundance RNAs in a sample and production of the corresponding cDNA, thereby, facilitating the cloning of low copy genes.

We used HCV and HIV RNA as templates for this purpose. The MMuLV RT is commercially available as SIII (mixture of RT and Taq polymerase) and hence was used directly while for the in-house purified RT (C-RT), a suitable amount of the commercial Taq DNA polymerase was included. A suitable control with plain Taq DNA polymerase was also run alongside. The end products were then tested on 3% agarose gel.

Briefly, the RT-PCR protocol followed for HCV was as follows. The reaction mixture of 25 µl consisted of 2X Superscript III buffer (ThermoFisher, USA). HIV and HCV PCR's were carried out independently in 25 µl reaction volumes. The PCR mixture comprised of 1 x Superscript III RT/Platinum® Taq Mix buffer, HIV (forward) primers 5-ATCAAGCAGCCATGCAAAT - 3′and reverse 5′-TACTAGTAGTTCCTGCTATGTC - 3′ that target the HIV-1 gag gene as described by Micheal et al. (1999) and HCV primers forward 5' GAA AGC GTC TAG CCA TGG CG 3' and reverse 5' ACG CCC AAA TGG CCG GGC ATA GA 3' as described by Rauf et al. (2010) with 0.5 µl of Superscript III and suitable amount of HCV RNA. The second set was carried out with suitable amounts of C-RT and 1 unit of Taq DNA polymerase as described by Sellner et al. (1992).

PCR was performed in a Proflex PCR machine (Applied Biosystems, USA). PCR conditions were cycles of 50 °C for 30 min to convert RNA to cDNA followed by single step of 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with a final extension of 72 °C for 10 min. For carrying out PCR's with Taq DNA polymerase alone, SIII was substituted by 1 unit of Taq DNA polymerase (Thermo Fisher, USA) keeping all other components and conditions same.

III. RESULTS

We have expressed the fragment comprising amino acid residues 80-273 of the MMuLV RT gene (Cote et al., 2008) using the expression vector, pET26b (Novagen, USA). The construct includes an N-terminal hexa-histidine tag and an enterokinase cleavage site. The protein was overexpressed in *E.* and purified after refolding from inclusion bodies using Ni-NTA (Novagen, USA) chromatography.

The plasmid map of pET26b-MMuLV-CRT (pCAN10) is given in Fig. 1A while Fig. 1 B shows the SDS-PAGE of the expression of recombinant CRT as insoluble inclusion bodies. The subcellular localization studies carried out showed the protein to be restricted to IB alone, and the recombinant RT was of expected size of 23 kDa (Fig. 1B).

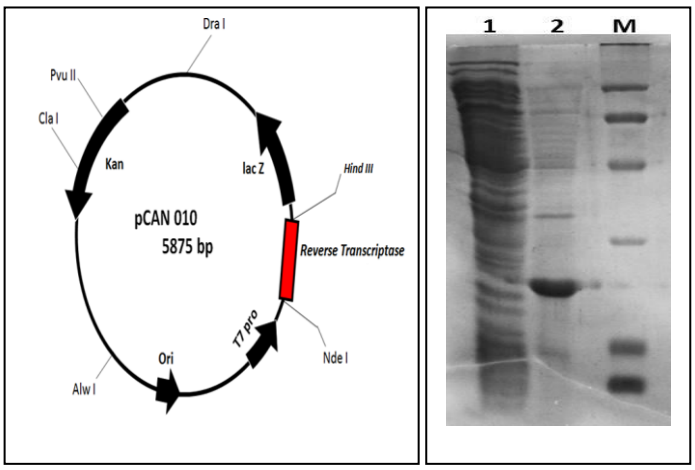

Fig. 1A: Plasmid Map of Pet26b-Mmulv-RT (C-RT), Designated as Pcan10, Used in this Study is Shown.

Fig. 1B SDS-PAGE showing expression of 23 kDa C-RT in ER2566 cells. The protein was found to be restricted to the insoluble fraction of the cell after sonication. Lane 1: soluble fraction; lane 2: Insoluble fraction showing C-RT protein. M: Protein molecular weight marker (14 kDa to 97 kDa).

The enzyme was found to be at least 95% pure as judged by silver stained gel, and the protein concentration was 23 mg/L of induced culture (Fig. 2).

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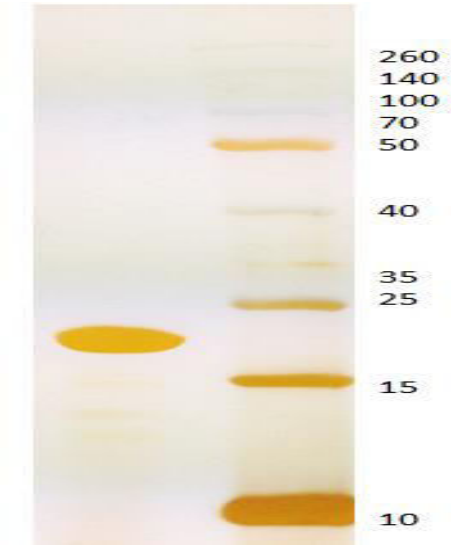

Fig. 2 SDS-PAGE Showing Purified C-RT by Silver Stained Gel**.** Note the purity of the purified RT is >95%. The sizes of the proteins of the molecular weight pre-stained marker (10 kDa to 260 kDa, Thermo Fisher Scientific, USA) are indicated.

Figure 3A shows that 6 of the 8 clinical samples tested showed strong HCV PCR signal with SIII enzyme; of

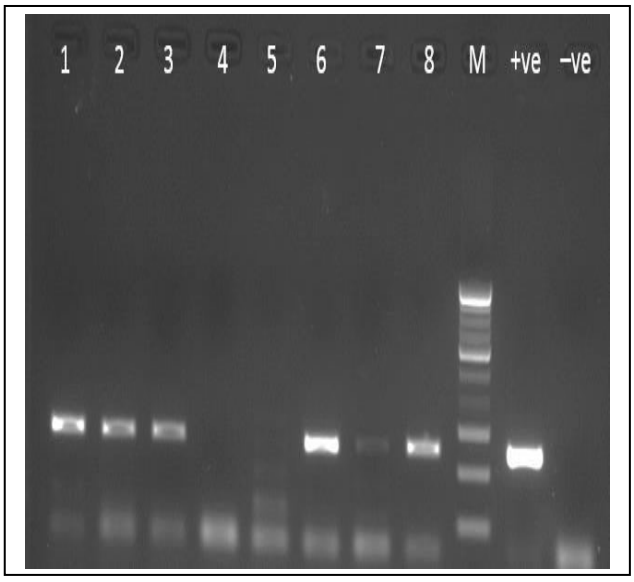

Fig. 3A Agarose Gel Showing PCR of HCV 5' UTR gene: The HCV RT-PCR was carried using gene specific primers as mentioned in M & M section. The samples #6, 7 and 8 were taken for activity studies with in-house purified C-RT

These 8 samples, 3 samples were randomly chosen for RT-PCR experiment using the in-house purified C-RT as the RT enzyme.

Similarly, of the 14 samples tested for HIV using HIV specific primers, 10 of them showed PCR signals for HIV as seen from the agarose gel electrophoresis. Three samples were randomly chosen for RT-PCR activity using C-RT preparation (Fig. 3B).

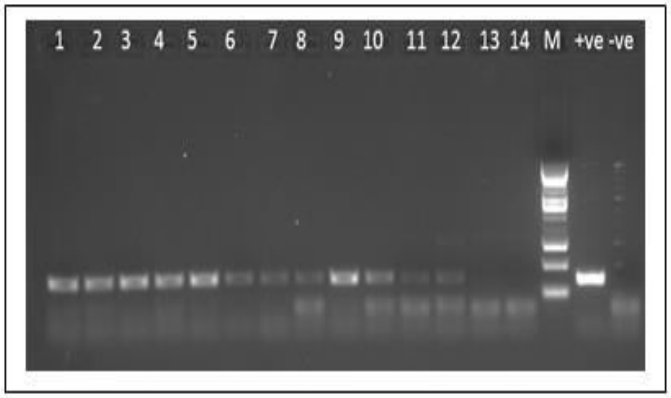

Fig. 3B Agarose gel showing PCR of HIV-1 gag gene: The HIV-1 RT-PCR was carried using gene specific primers as mentioned in M & M section. The samples #6, 7 and 8 were taken for activity studies with in-house purified C-RT.

It is evident from Figure 4 that HCV signals for two batches of C-RT purified in-house gave strong signals for HCV as shown in the Agilent Bioanalyzer 2100 electropherogram, reflecting the reproducibility of the purification protocol followed. The sample #7, which showed faint signal with SIII, also showed similar intensity of the band when the reaction was carried out with C-RT. This indicates that the efficiency of the in-house purified C-RT is similar to the commercially available SIII enzyme.

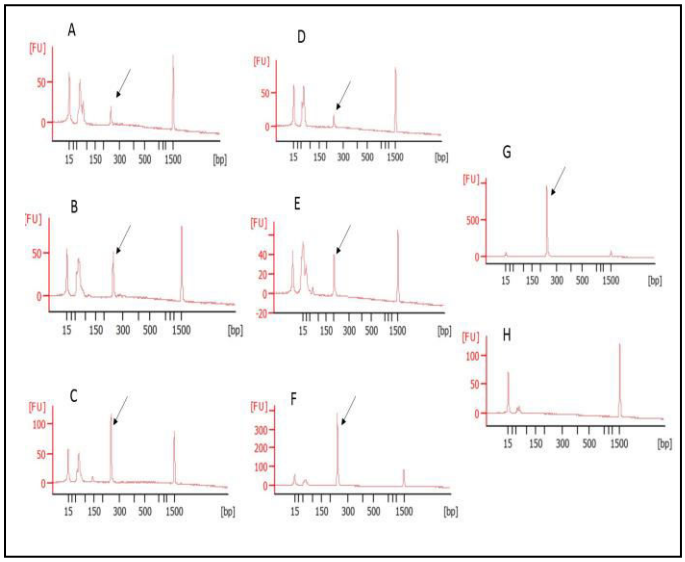

Fig. 4 Agilent electropherograms showing HCV PCR signals using in-house C-RT and commercial SIII enzymes. Panel A: with clinical sample #5 RNA and in-house purified RT; panel B: with clinical sample #6 RNA and in-house purified RT; panel C: with clinical sample #7 RNA and in-house purified RT; panel D, E and F show electropherograms with SIII for the RNA from the clinical samples #6, #7 and #8 respectively. Panel G and H indicate PCR signals obtained with positive control (PCR amplicon of HIV-1 gag gene as template) and no template control respectively. Note the similar intensity of the PCR signals of all clinical samples achieved using in-house RT and SIII. Arrows denote the peak of the HCV PCR signal. Note that there is no peak seen with no template control demonstrating the specificity of the PCR reactions employed in this study (panel H).

The agarose gel showing HCV signals for the same samples is shown in Fig. 5.

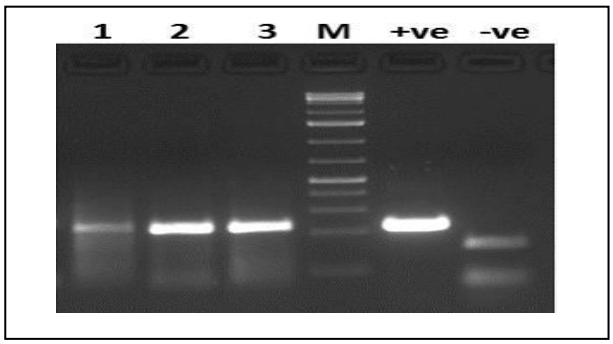

Fig. 5 Agarose gel electrophoresis showing HCV PCR signals for three random RNA samples taken for activity assay using in-house purified C-RT.
Lane 1: with sample #7 ; lane 2: sample #6, lane 3: with sample #8. M: DNA molecular weight marker; + ve: with positive HCV PCR amplicon as template; -ve: no template PCR control

From the data presented in Fig. 6, it is clear that the in-house purified RT showed signals for HIV target gene, indicating that the protocol employed for purification of the C-RT is successful.

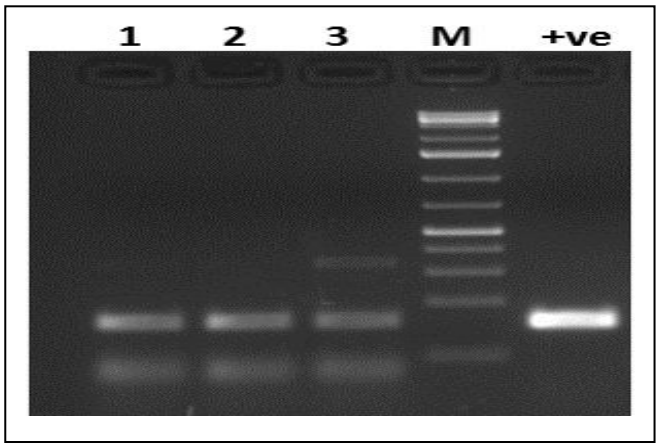

Fig. 6 Agarose gel electrophoresis showing HIV-1 PCR signals for three random RNA samples taken for activity assay using in-house purified C-RT.
Lane 1: with sample #10; lane 2: sample #11, lane 3: with sample #12; M: DNA molecular weight marker; + ve: with positive HIV-1 PCR amplicon as template.

IV. DISCUSSION

The polymerase gene of MuMLV coding for RT has been cloned into a prokaryotic expression vector with lambda phage promoter, and it represents 20% of the total cellular protein (Kotewicz et al., 1985). Various purification protocols have been developed and published for purification of full-length MMuLV RT. These include sequential purification over anion exchange, phosphocellulose, DNA binding column matrix, and cation exchange columns (Arezi and Hogrefe, 2009). Chen et al. (2009) have reported a novel protocol for producing recombinant MMLV-RT in *E. coli*. In their study, the optimized coding sequence for mature MMLV-RT was over-expressed as an N-terminal His-tagged fusion protein with an enterokinase (EK) recognition site using chaperone co-expression and lower temperature fermentation. Nearly 21 mg MMLV-RT was obtained from 1 l of bacterial culture.

Fei et al. (2012) reported the point mutation L432K, V433K, I434K, L435K and A436K made by PCR-based site-directed mutagenesis. This enabled them to get a soluble expression of the full-length RT protein. However, they did not mention the yield of the protein per liter of the culture. Similarly, Arezi and Hogrefe (2009) identified five mutations (E69K, E302R, W313F, L435G, N454K) that collectively increased the half-life of M-MuLV RT at 55 ^{o}C from 5 min to 30 min in the presence of the template-primer. It is well known that RT inhibits Taq DNA polymerase activity and that inactivated RT fails to inhibit Taq polymerase (Sellner et al. (1992). Hence, protocols in which an RNA template is successfully processed by RT followed by PCR with Taq polymerase would show positive PCR signals. The results of positive RT-PCR signals with in-house C-RT indicate that the protocol described for refolding and purification of MMuLV RT yields active protein and can be successfully applied in large scale.

Roth and co-workers (1985) have purified the full-length MMuLV-RT using DEAE-cellulose, phosphocellulose, polyribocytidylic acid-agarose, and hydroxylapatite columns with merely 8% yield from the starting material. Ammonium sulfate followed by phosphocellulose was employed by Tanese et al. (1985) while Baranauskas et al. (2012) used Ni affinity followed by phosphocellulose to achieve 90% purity of the full-length enzyme with no mention of the total protein recovery.

Sun et al. (1998) have reported the expression and purification of the catalytic fragment of the MMuLv RT of 28 kDa, from 24-278 amino acids from MMLV RT under a T7 expression vector (pET15b) with N-terminal hexa-histidine tag and thrombin cleavage site and obtained 10 mg of pure protein after multiple steps of purification. Since some of the C-terminal insertions are observed to render the polymerase activity thermolabile and one such insertion nearly abolished that activity, it was concluded that there are interactions between these regions.

The only paper that speaks about purification of a viral RT by denaturation and refolding is of an RT from HIV by Deibel et al. (1990). The protocol described in this paper merely gave 4% yield of the active protein. Other purification protocols utilizing various denaturing conditions like guanidine hydrochloride, 8 M urea at pH 8.0 with and without arginine, purification of the RT under denaturing conditions on the Ni-NTA column were also evaluated for yielding homogeneous, active preparations. But these protocols showed limited success. Hence, the protocol described here with a yield of ~23 mg/l of the purified RT is the highest yield for an RT recovered from inclusion bodies to date and thus, appears attractive and scalable.

Recombinant proteins expressed as inclusion bodies have several advantages in comparison to the proteins expressed as soluble entities in the cytoplasm of an expression like *E. coli*. The advantages include reduced fermentation cost, reduced amount of cytoplasmic proteins, negligible host endonucleases and nucleic acids, and proteins that are toxic to the expression host can be safely expressed as inclusion bodies. The high degree of purity of the target protein protects the protein from proteolytic degradation compared to the soluble counterpart.

High-level expression of many recombinant proteins in prokaryotic system as inclusion bodies are usually recovered from bacterial cell lysates after cell lysis by sonication, by using ultrasonication for small, French press for medium, or high-pressure homogenization for large-scale cell lysis (Vallejo and Rinas, 2004). Such inclusion bodies are processed by selective extraction with detergents like Triton X-100 and low concentrations of either urea or Gu·HCl to produce the so-called washed pellets to remove *E. coli* cell wall and outer membrane components, removal of membrane proteins or other non-specifically adsorbed cell material (Georgiou and Valax, 1999; Clark et al., 1999; Palmer and Wingfield, 2004).

Strategies for recovering active proteins from bacterial inclusion bodies for proteins with disulfide bonds are well reported. The disulfide bonds are more efficiently formed when a mixture of low molecular weight thiols (e.g. glutathione) in their reduced and oxidized state is added to the refolding buffer (Ahmed et al., 1975). The best conditions for refolding of disulfide-bonded proteins are the excess presence of the reduced form and slightly alkaline pH. These conditions allow rapid disulfide exchange reactions until the protein reaches the most stable disulfide-bonded configuration, i.e., the native state of the protein. MMLV-RT is poorly soluble and prone to molecular aggregation, making them biologically inactive (Tanese et al., 1985).

The protocol described in this article utilizes endA1 minus *E. coli* host such as ER2566 for expression of the recombinant RT, and hence, our purified RT would be free of any endonuclease contamination, which is useful and cost-advantageous for its intended use in PCR-based diagnostics. This is because it has been suggested that contaminated endonuclease activity in recombinant protein preparations results from expression of the native *E. coli* endA gene (Singleton et al., 2002). The limited solubility of MMLV RT is a major difficulty in mass enzyme production in the prokaryotic system. However, our results on a simple refolding protocol to achieve active MMuLV RT from insoluble inclusion bodies is encouraging and opens up new avenues of trying this protocol in large scale.

M-MLV RT RNase H– point mutant is the enzyme of choice for reverse transcribing long RNAs for cDNA library construction, cDNA probe generation and primer extension due to its lack of RNase H activity. Hence, the work reported on an RT without the RNaseH domain assumes critical importance. Invitrogen's SuperScript III Reverse Transcriptase is a genetically engineered MMLV RT that was created by the introduction of several mutations for reduced RNase H activity, increased half-life, and improved thermal stability. Other mutations have also been found to increase the intrinsic stability of H- MMuLV RT without affecting template binding; however, their identities have not been disclosed (e.g. SuperScript III RT, Invitrogen). Our data demonstrating the similarity in enzyme efficiency of the in-house RT to the commercially available Superscript III from Invitrogen is encouraging and in the future, we intend to carry out certain mutations in this molecule and look for enhanced thermostability of the C-RT preparation.

V. ACKNOWLEDGMENT

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