MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society for Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

# EXPRESSION OF RECOMBINANT HUMAN EXONUCLEASE1 (HEXO1) FROM BACTERIA

Umar A. A[1*]., Fakai, I. M[1] & Bagudo, A. I[2]

[1]*Department of Biochemistry, Kebbi State University of Science and Technology, Aliero, P.M.B 1144 Birnin Kebbi, Nigeria*
[2]*Department of Microbiology, Kebbi State University of Science and Technology, Aliero, P.M.B 1144 Birnin Kebbi, Nigeria*

*Corresponding Author: auargungu96@gmail.com

**SHORT COMMUNICATION**

History
Received: 27th July 2019
Accepted: 22nd December 2019

Keywords:

*hExo1; BL21-CodonPlus; pTXB1*

**Abstract**

Human exonuclease1 (hExo1) directly involves in many events of DNA processing such as replication, mismatch repair (MMR), and double strand break repair (DSBR), and can also act as a sensor to lesion and inducer of apoptosis in the event of DNA lesion. Human Exo1 protein was functionally expressed in E. coli strain BL21-CodonPlus (DE3)-RIL. Expression of hEXo1 in E. coli under the transcriptional regulation of the T7 promoter yielded a soluble cytosolic monomeric protein with an apparent molecular mass of 110 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Recombinant hExo1 was purified to near homogeneity using a two-step purification procedure that involves affinity chromatography using heparin column and size-exclusion chromatography. A yield of about 100 µg of the hExo1 protein could be purified from 500 mL of E. coli strain BL21-CodonPlus (DE3)-RIL cells. The purified protein was analysed by LC-ESI-tandem MS on a Q-TOF2 mass spectrometer fitted with a nanoflow ESI (electrospray ionization) source and confirmed to be hExo1.

## INTRODUCTION

Human exonuclease1 (hExo1) is a multifunctional nuclease that plays important functions in DNA processing events such as mismatch repair (MMR), double stranded break repair (DSBR) as well as in recombination during meiosis and mitosis. The full length hExo1 has been reported to function in MMR, recombinational repair, homologous recombination (HR) and DNA end resection [1]. Exo1 was first discovered in *Schizosaccharomyces pombe* [2] as a member of proteins family called Rad2/XPG, this family of proteins are characterized of having a highly-conserved active domain located at the N-terminal region of the protein [3]. Exo1 possesses 5'→ 3' exonuclease, 5' structure-specific DNA endonuclease, and 5'→3' RNase H activity [4,5,6]. Exo1 has high affinity for processing double-stranded DNA, nicks, gaps and pseudo-Y structures and it can resolve the double Holliday junctions [4,6,7, 8]. The contribution of Exo1 in safeguarding the stability of the genome during DNA replicative and post-replicative processes is well-established. Attempts to express and purify the full-length human exonuclease1 protein from bacteria have proven difficult, largely due to rapid degradation [3,5]. Nearly all current biochemical studies on the full-length human hExo1 expressed the recombinant protein in insect cells [9-12]. This work reported an *E.coli* based expression system to expressed and purify human hExo1.

## MATERIALS AND METHODS

### Materials

The full-length clone for the human Exo1 splice variant (*hExo1* cDNA) in a pFastBacI plasmid (pFastbacI-hExo1) was provided by Professor Paul Modrich, (HHMI & Department of Biochemistry, Box 3711, Rm 159 Nanaline Duke Bldg Duke University Medical Centre Durham NC 27710, 919-684-2775). All restriction enzymes were purchased from New England Biolabs. All antibiotics were purchased from Sigma Aldrich. IMPACT™ Kit (contained pTXB1 vector) was purchased from New England Biolabs GeneJET Plasmid Miniprep Kit was purchased from Fisher Thermo Scientific.

### Methods

***Cloning of hExo1 into pTXB1 vector:*** The coding region of hExo1 (codons 1-846) was amplified by PCR using a full-length clone for the human Exo1 splice variant (*hExo1* cDNA) in a pFastBacI plasmid (pFastbacI-hExo1) as template and two synthetic oligonucleotides, 5'-GGGAATTCCATATGGGGATACAGGGATTGCTA-3' and 5'-AAGGCCGCTCTTCCGCACATCTGGAATATTGCTCTTTGA ACACGG-3' as forward and reverse primers, which contain *NdeI* and *SapI* recognition sequences overlap (underlined) at their 5' ends, respectively. A 10 µL of the finished PCR reaction was

removed and analysed by 1% agarose gel electrophoresis to check the fragment size. PCR product was then purified away from the PCR components using the PureLink™ PCR clean-up/Quick Gel Extraction System (Invitrogen). The purified PCR product was digested using *NdeI* and *SapI* and inserted between the *NdeI* and *SapI* sites of the pTXB1 plasmid (New England Biolabs) to construct a gene fusion between hExo1 and a self-cleaving affinity tag that is composed of an Mxe intein fragment and the chitin-binding domain (El-Shemerly, *et al* 2005).

Competent *E. coli* DH5α cells were transformed by adding an aliquot of 5 µL ligation reaction and heat-shocked on a heating block at 42ºC for 30 seconds then placed immediately onto ice. About 250 µL of pre-warmed SOC media was added into the tube and incubated at 37ºC for 60 minutes so that the cells can recover. The culture was plate out on LB agar containing 150 µg/ml ampicillin and 25 µg/ml chloramphenicol then incubated at 37ºC overnight. The next day, about 10 colonies were selected, circled and numbered on the plate to take note of their identity. Each of the selected colonies was touched with a sterile pipette tip and agitated in 0.5 ml tube containing 20 µL $dH_2O$ and heated at 100ºC on a heating block for 5 minutes and then pulse-centrifuged. An aliquot of 3 µL supernatant was transferred to PCR tube and set up the PCR reaction. The PCR product was analysed on 1% agarose gel to identify for the correct fragment. Once the correct fragment was identified the colony that contained such a fragment was selected for miniprep. GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used to purify the recombinant plasmid according to manufacturer's protocol. Purified sample which contained the recombinant plasmid DNA (pTXB1-hExo1) was stored at -20ºC. The construct was then verified by sequencing (Eurofins Genomics).

**Expression of hExo1 protein from bacteria**

*E. coli* strain BL21-CodonPlus (DE3)-RIL (Agilent technologies) was transformed with the pTXB1 derived *hExo1* construct for protein expression. From a single colony of transformed *E. coli* cells on LB agar plate a 50 mL LB broth containing 150 µg/ml ampicillin and 25 µg/ml chloramphenicol starter culture was inoculated using flame sterile technique. The 50 mL starter culture was transferred to a 500 mL LB broth containing 150 µg/ml ampicillin and 25 µg/ml chloramphenicol then allowed to grow to an $OD_{600}$ = 0.3 at 37ºC with rotation of 250 rpm, then protein expression was induced by the addition of 0.2 mM IPTG and the incubator temperature was adjusted to 18ºC. The culture was allowed to grow overnight with rotation of 250 rpm. The following day, cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4ºC in Beckman Coulter centrifuge using JA-10 rotor. Cell pellets were re-suspended in 50 ml pre-chilled (4ºC) CH buffer (20 mM Tris pH 8.0, 500 mM NaCl, 0.1% v/v Triton X100, 1 mM EDTA, 10% v/v glycerol) supplemented with 0.1 mM PMSF and Roche complete inhibitor cocktail.

**Purification of recombinant hExo1 protein**

Protein purification was conducted as described previously but with some modifications [13]. The bacterial suspension was sonicated by a continuous sonication on ice, 1 min at maximum power followed by 1 min incubation on ice, this was repeated 5 times and PMSF was added after sonication. Sonicated bacterial suspension was centrifuged at 18000 rpm for 45 minutes at 4ºC in JA-20 rotor. The cleared supernatant containing the fusion protein of interest was saved at 4ºC. A 10 mL of chitin-beads suspension (New England Biolabs) an affinity matrix for the isolation of target proteins fused to an intein-chitin binding domain fusion was poured in a frit-equipped plastic column (Bio-Rad) and allowed the storage solution to drain by gravity flow, the column was equilibrated by passing 100 ml of CH buffer (20 mM Tris pH 8.0, 500 mM NaCl, 0.1% v/v Triton X100, 1 mM EDTA, 10% v/v glycerol) + PMSF by gravity flow. About 50 ml soluble fraction containing hExo1-Intein was loaded onto the column and allowed to flow slowly (~ 0.5 ml/min) by gravity flow, the column was washed with 200 mL CH buffer + PMSF. The on column proteolytic cleavage of the Intein tag was then triggered by closing the tube and 5 mL of CH buffer + PMSF + 30 mM DTT was poured into the column and stirred gently with a sterile glass rod. This was allowed to incubate over night at 4ºC. The next day the column was opened and the first 5 mL fraction containing hExo1 was recovered by gravity flow using a tube placed on ice, another 5 mL CH buffer + PMSF was poured in the column and recover a second 5 mL fraction, this step was repeated until 5 fractions were recovered. All the eluted fractions were pooled together and concentrated down using spin-X UF concentrator (Corning) and passed through a Superose 12 10/300 (GE Healthcare Life Sciences) gel filtration column using an AKTA prime protein purification system, column was pre-equilibrated with buffer (20 mM HEPES pH 7.5, 100mM KCl, 10% v/v glycerol) and the column washed with the same buffer. After gel filtration, hExo1 containing fractions were pooled together and concentrated with Spin-X UF concentrator (Corning) and snap-frozen in liquid nitrogen, then stored at -80ºC.

**Mass spectrometry**

The identity of the purified recombinant protein was verified by mass spectrometry. Sample was analysed by LC-ESI-tandem MS on a Q-TOF2 mass spectrometer fitted with a nanoflow ESI (electrospray ionization) source (Waters Ltd). Peptides were delivered on-line to the MS via a CapLC HPLC system, with peptide trapping on a C18 PepMap100 (5 µm, 100 Å) µ-Precolumn 300 µm i.d., 5 mm (Thermo).

## RESULTS AND DISCUSSION

**Construct for hExo1 protein expression in E. coli cells**

To construct a translational fusion between hExo1 and an affinity tag that is composed of an Mxe intein fragment and the chitin-binding domain, the coding region of hExo1 (codons 1-846) was amplified by PCR and the PCR product was inserted between the *NdeI* and *SapI* sites of the pTXB1 plasmid (NEB). The construct was first verified by agarose gel analysis which revealed that the pTXB1+hExo1 construct has produced a larger plasmid ~8.6 kb compared to pTXB1 vector alone ~6.3 kb indicating that the insert has been ligated (**Figure 1**, lane 4). To further verify, the construct was sent for sequencing (Eurofins Genomics, Germany). The sequencing result matched NCBI sequence ID ref NC_000001.11 with 100% query cover, 0.0 E values and 100% identity, with features as exonuclease 1 isoform b.

When incubated in either the presence or the absence of IPTG, *E. coli* BL21-CodonPlus (DE3)-RIL harbouring the pTXB1 derived *hExo1* construct over-expressed hExo1, as determined by analysis of cell lysates for the presence of a Coomassie blue-stainable protein. The recombinant hExo1 was detected in the soluble fraction of cell lysates (**Figure 2**A, lane 3) which indicated that the protein was expressed as soluble cytosolic monomeric protein with an apparent molecular mass of 110 kDa.

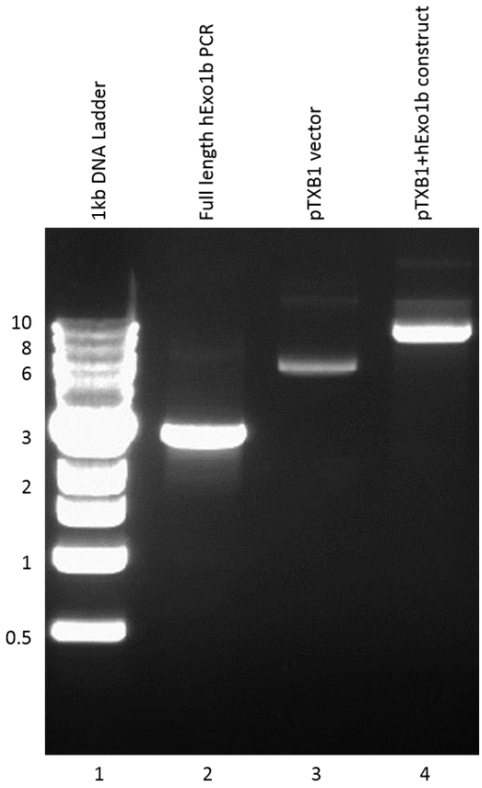


**Figure 1.** Agarose gel analysis for pTXB1+hExo1 construct. A 100 ng PCR product lane 2, 50 ng vector lane 3 and 100 ng pTXB1+hExo1 construct lane 4.

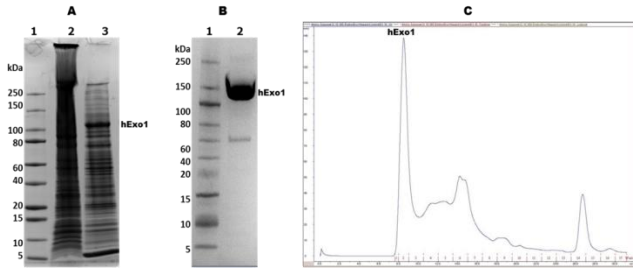


**Figure 2.** SDS/PAGE analysis for the bacterial expression of full length hExo1 protein (A) SDS/PAGE analysis of the whole cell lysate lane 2 and the soluble cell lysate lane 3. (B) SDS/PAGE analysis of the purified recombinant hExo1 (C) Chromatogram profile of hExo1 after size-exclusion chromatography using Superose 12 10/300 column.

**Purification of hExo1 protein**

Human Exo1 was purified to near homogeneity using a strategy previously described [10,13] but with some minor modifications that were found to be essential to consistently obtain pure protein. Human Exo1 has a molecular weight of 94 kDa, but migrated similar to proteins of about 110 kDa in size in the presences of sodium dodecyl sulfate (Figure 2b, lane 2), this migration behaviour of hExo1 was observed in previous studies [1,11,13,14]. This is most likely due to the unusual amount of charged amino acids found in the intrinsically disordered portion of the protein that have adverse effects on the amount of SDS bound to the protein during electrophoresis and hence lead to anomalous migration of the protein. Gel filtration chromatography of the chitin resin purified sample containing hExo1 showed that hExo1 eluted near the void volume (Figure 2c). After gel filtration chromatography, fractions where the chromatogram peak appeared with higher intensity were collected and analysed by SDS/PAGE.

**Mass Spectrometry**

Protein Lynx Global Server version 2.0 (Waters, Ltd) was used to process the uninterpreted MS data into peak list (pkl) files which were searched against all entries in the NCBInr database (version 20140228) using the web version of the MASCOT MS/MS ions search tool (http://www.matrixscience.com/). Recombinant protein was confirmed from the data search results as human exonuclease1. Human Exo1 is predicted to have three main disordered regions located in the C-terminal region. In fact, about two-thirds of the hExo1 is predicted to be disordered. Recombinant expression of most disordered proteins is challenging since significant active protein yields are required and because these proteins should be in a native and functional conformation during expression and purification. The natural abundance of most intrinsically disordered proteins is typically not high enough for the isolation of sufficient quantities for functional and structural studies. Attempts to express and purify the full-length human exonuclease1 protein from bacteria have proven difficult, largely due to rapid degradation [3,5]. Nearly all current biochemical studies on the full-length human hExo1 expressed the recombinant protein in insect cells [9-12]. This work reported an *E.coli* based expression system to expressed and purify human hExo1. The new system of producing recombinant full-length human hExo1 will be valuable for researchers in the field.

**ACKNOLEDGEMENTS**

We thank Paul Modrich for human Exo1 expression construct.

**CONFLICT OF INTEREST**

There is no any conflict of interest among the authors in whatever form.

**REFERENCES**

1 Genschel J., Bazemore L., Modrich P. (2002): Human exonuclease I is required for 5' and 3' mismatch repair, J. Biol. Chem. (277) 13302–13311.

2 Szankasi P, Smith GR. (1995). A role for exonuclease I from *S. pombe* in mutation avoidance and mismatch correction. *Science (New York, N.Y.)* 267:1166–9.

3 Wilson, D. M., III, Carney, J. P., Coleman, M. A., Adamson, A. W., Christensen, M., and Lamerdin, J. E. (1998) *Nucleic Acids Res.* **26,** 3762–3768

4 Lee B., Wilson D. R. (1999): The RAD2 domain of human exonuclease 1 exhibits 5_ to 3_ exonuclease and flap structure-specific endonuclease activities, J. Biol. Chem. (274) 37763–37769.

5 Qiu J., Qian Y., Chen V., Guan M.X., Shen B. (1999): Human exonuclease 1 functionally complements its yeast homologues in DNA recombination, RNA primer removal, and mutation avoidance, *J. Biol. Chem*. (274) 17893–17900.

6 Keijzers G, Bohr VA, Rasmussen LJ. (2015). Human exonuclease 1 (EXO1) activity characterization and its function on flap structures. *Biosci Rep* 35:e00206.

7 Genschel J., Modrich P. (2003): Mechanism of 5'-directed excision in human mismatch repair, Mol. Cell (12) 1077–1086.
8 Zakharyevich K, Ma Y, Tang S, et al. (2010). Temporally and biochemically distinct activities of Exo1 during meiosis: double-strand break resection and resolution of double Holliday junctions. *Mol Cell* 40:1001–15.
9 Nimonkar, A.V., Genschel, J., Kinoshita, E., Polaczek, P., Campbell, J.L., Wyman, C., Modrich, P. And Kowalczykowski, S.C. (2011) BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. *Genes Dev*. 25, 350–362.
10 Andersen, S.D., Keijzers, G., Rampakakis, E., Engels, K., Luhn, P., El-Shemerly, M., Nielsen, F.C., Du, Y., May, A., Bohr, V.A. *et al*. (2012) 14-3-3 checkpoint regulatory proteins interact specifically with DNA repair protein human exonuclease 1 (hEXO1) via a semi-conserved motif. *DNA Repair* (Amst.) 11, 267–277
11 Chen, X., Paudyal, S.C., Chin, R.I. and You, Z. (2013) PCNA promotes processive DNA end resection by Exo1. *Nucleic Acids Res*. 41, 9325–9338
12 Yang S.H., Zhou R., Campbell J., Chen J., Ha T. And Paull T.T. (2013) The SOSS1 single-stranded DNA binding complex promotes DNA end resection in concert with Exo1. *EMBO J*., 32, 126–139
13 El-Shemerly M, Janscak P, Hess D, et al. (2005). Degradation of human exonuclease 1b upon DNA synthesis inhibition. *Cancer Res* 65:3604–9.
14 Bregenhorn,S. and Jiricny,J. (2014) Biochemical characterization of a cancer-associated E109K missense variant of human exonuclease 1. *Nucleic Acids Research* **42**, 7096–7103.