Title:

A nonradioactive restriction enzyme-mediated assay to detect DNA repair by Fe(II)/2-oxoglutarate dependent dioxygenase

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Abstract:

The *Escherichia coli* DNA repair enzyme AlkB belongs to Fe(II)/2-oxoglutarate-dependent dioxygenase family. It removes methyl groups from 1-methyl Adenine (1-meA) and 3-methyl Cytosine (3-meC) lesions present in single-stranded DNA by oxidative decarboxylation. In the present paper we describe an *in vitro* assay that permits rapid detection of AlkB activity. To achieve this, we generated methylated oligonucleotide using methyl methanesulfonate and then monitored DNA repair using a methylation sensitive restriction enzyme and novel agarose gel electrophoresis system capable of resolving small oligonucleotides. Our approach overcomes several drawbacks of NAD^+-dependent formaldehyde dehydrogenase-coupled assay and radioisotope-based assay for determining AlkB DNA repair activity.

Keywords:

Fe(II)/2-oxoglutarate-dependent dioxygenase, AlkB, Methyl methanesulfonate, DNA repair

Alkylating agents constitute an important class of DNA damaging chemicals present in the environment and also generated endogenously as byproducts of cellular metabolism. [1]. Alkylating agents cause several types of DNA adducts, depending on the mode of the chemical reaction. Members of a large family of Fe(II)/2-oxoglutarate-dependent dioxygenases directly repair these lesions by oxidative decarboxylation involving hydroxylation of alkyl group and its removal as aldehyde [2; 3]. *Escherichia coli* alkylation repair protein-B (AlkB, EC 1.14.11.33) is the most well characterized member of this family which repair 1-methyl Adenine (1-meA) and 3-methyl Cytosine (3-meC) lesions present in single-stranded DNA (ssDNA) [4]. AlkB homologues are present in most organisms including human [5; 6]. However, very little is known about their cellular function in DNA repair. Lack of a convenient and rapid *in vitro* biochemical assay further limited the study of DNA repair activity of the AlkB homologues. Although generic assays based on monitoring of the substrate depletion or the product formation using an NAD^+-dependent formaldehyde dehydrogenase-coupled assay was reported [7], traditional method for determining AlkB DNA repair activity was carried out with DNA substrates modified by [^{3}H]-methyl methanesulfonate (MMS) or [^{14}C]-methyl iodide. DNA Repair was detected by quantifying residual radioactive methylated nucleotides in the substrate by HPLC analysis [8]. Another DNA repair assay based on restriction digestion of methylated DNA was described by Wu *et al* [9] and later modified by Ringvoll *et al* [10] and Westbye et al [11]. This assay method included chemical synthesis of 1-meA and 3-meC and their incorporation within oligonucleotides. The modified oligonucleotides were then end-labeled at the 5'-terminus using radioactive tag [γ^{32}P]-ATP or fluorescent tag carboxyfluorescein. Removal of the methyl group was assayed by restriction digestion using 20% denaturating PAGE and autoradiography. Limitations to this assay include tedious protocol, requirement for chemical synthesis of modified oligonucleotide substrate and fluorescent or radioactive labeling.

In order to simplify the *in vitro* DNA repair assay for AlkB like Fe(II)/2-oxoglutarate-dependent dioxygenases we first established a method to generate methylated substrate. We designed a 70bp oligonucleotide with an MboI restriction site at the middle such that digestion with MboI would result two 35bp fragments (Fig1A). Desalted oligonucleotides were purchased from Sigma-Aldrich. Sequence of the oligonucleotide selected for methylation was 5'-GGATGCCTTC GACACCTAGC TTTGTTAGGT CTGGATCCTC GAAATACAAA GATTGTACTG AGAGTGCACC-3'. Since MboI digestion is inhibited by DNA methylation including 1-meA and 3-meC, restriction digestion of methylated oligonucleotide would not yield any 35bp fragments (Fig1B). However, if AlkB repairs this methylated substrate then it would become susceptible to MboI digestion. In other words, MboI digestion of methylated substrate would indicate successful DNA repair. We generated methylated nucleotide by treating the oligonucleotide with MMS *in vitro*. It was reported earlier that MMS treatment of ssDNA predominantly yields 7-methyl Guanine (7-meG), 1-meA and 3-meC [12]. Among these lesions 1-meA and 3-meC are cytotoxic as they block replication. However, MMS being a methanesulfonic acid methyl ester, direct incubation of DNA with MMS resulted acidic pH and DNA degradation. Thus, methylation reaction was performed in presence of dibasic phosphate buffer to neutralize the pH of the reaction mixture. The standard methylation reaction involved incubation of 40μg of 70 nucleotide ssDNA oligonucleotide with 5% (0.59M) MMS (stock prepared in 50% ethanol), 200mM K_2HPO_4 in 500μl of total reaction for 170 min at room temperature. The methylated ssDNA could not be purified directly by using ethanol precipitation as presence of residual MMS caused an insoluble precipitate. Therefore, MMS was removed by dialysis against TE buffer (10mM Tris. pH 8.0, 1 mM EDTA) using Spectra/Por dialysis membrane (MWCO: 3,500). Then the damaged substrate was precipitated by adding 0.3M sodium acetate pH 5.5 and 2 volume of ice-cold ethanol. The precipitated ssDNA was washed with 70% ethanol and

finally dissolved in water. Methylated double-stranded DNA containing the methylated strand was prepared by annealing a desalted oligonucleotide with complementary DNA of sequence 5'-GGTGCACTCT CAGTACAATC TTTGTATTTC GAGGATCCAG ACCTAACAAA GCTAGGTGTC GAAGGCATCC-3' with the prevously described methylated oligonucleotide. While the standard method for annealing complementary oligonucleotides involves heating the complementary oligonucleotides at 95°C for 2-5 minutes followed by gradual cooling to room temperature this could not be applied for annealing the methylated oligonucleotides as the alkylated bases led to the generation of apurinic/apyrimidinic sites and subsequently strand break [13; 14]. Therefore, annealing of methylated oligonucleotide (6.5µg) and unmethylated complementary oligonucleotide (4.5µg) was carried out at 37°C for 30 min in the presence of annealing buffer (50mM HEPES, pH 8.0, 10mM EDTA) in 15µl reaction. We developed simple approach to avoid radioactivity or fluorescence-based labeling and denaturating PAGE to analyze the MboI digested oligonucleotides. We used a novel electrophoresis buffer that allowed analysis of the oligonucleotides on agarose gel. Oligonucleotides were resolved on a 3% agarose (Lonza) gel containing 0.1µg/ml ethidium bromide with 10 mM Sodium Borate (SB) as electrophoresis buffer at 300V for 20 min as previously reported [15]. The annealed 70 base pair (bp) double-stranded oligonucleotides exhibited a clear band (as shown in Fig 1C, Lane 1). The mobility of this band could be confirmed by matching with Ultra Low Range (Thermo Scientific) molecular size marker (Fig1C, Lane 1). ssDNA showed faster mobility than the double-stranded DNA (Fig1C, Lane 2). To check if the substrate annealing was correct, 70bp double-stranded oligonucleotide (3µg) was treated with MboI (New England Biolabs) for 2h and analyzed by agarose gel electrophoresis. The 70bp double-stranded oligonucleotide (Fig 1D, Lane 2) was completely cleaved by MboI and resulted 35bp fragment (Fig 1D, Lane 3). Methylated 70bp double-stranded oligonucleotide, generated by

annealing the MMS-treated ssDNA with the complementary unmodified strand, was also analyzed by agarose gel analysis (Fig 1D, Lane 4). However, MMS-treated 70bp methylated double-stranded DNA (3µg) was not cleaved by MboI restriction endonuclease due to the presence of 1-meA and 3-meC modification (Fig 1D, Lane 5).

After confirming that MMS-treated DNA was resistant to MboI digestion, it was expected that AlkB mediated oxidative demethylation of the MMS-treated DNA might render it susceptible to restriction digestion by MboI. To examine this hypothesis recombinant AlkB was produced in *E. coli* by cloning AlkB gene into pGex-6p1 expression vector (GE-healthcare) at BamHI and SalI sites. To verify specificity of oxidative demethylation reaction by AlkB, two more *E.coli* dioxygenases, namely, *mhpB* gene, encoding catechol dioxygenase [16] and *tauD*, encoding taurine hydroxylase [17], were cloned into the same vector. GST tagged MhpB, TauD and AlkB were purified using Glutathione Sepharose 4-FastFlow (GE-healthcare) (Fig 2A, Lanes 2-5). *In vitro* DNA repair was carried out at 30°C for 4h by each of these proteins (2.4 µM) and MMS-treated ssDNA in a reaction (25µl) containing repair buffer (2mM HEPES, pH 8.0, 20 µM 2-oxoglutarate, 0.2mM L-ascorbic acid, 2µM $Fe(NH_4)_2(SO_4)_2$ and 100µg/ml BSA). Following repair of the damaged ssDNA, the complementary strand was annealed. MboI digestion of AlkB-treated methylated DNA resulted 35bp fragment (Fig 2B, lane 5). However, MboI cleavage was absent in TauD treated sample (Fig 2B, lane 4). TauD, a Fe(II)/2-oxoglutarate-dependent dioxygenase family member involved in degradation of taurine (2-aminoethanesulphonic acid), failed to repair methyl adducts from DNA. This result suggests that the demethylation of damaged DNA function is specific to AlkB function. Similarly, neither bacterial dioxygenase MhpB nor GST showed any repair activity (Fig 2B, lanes 2 and 3). After confirming the specificity of AlkB mediated DNA repair, *in vitro* repair was carried out by incubating increasing concentration (0.4-2.4 µM) of recombinant AlkB, with the MMS-

treated ssDNA. After continuing the reaction at 30°C for 4h the complementary strand was annealed. As shown in Fig 2B, lane 2, 35bp DNA fragment was absent when AlkB was omitted from the reaction. However when higher concentration of AlkB was used 35bp fragment appeared (Fig 2C, lanes 3-5) indicating that AlkB successfully removed methyl groups in a concentration-dependent manner (Fig 2D). Next, we monitored the continuous repair of the methylated DNA by annealing the repaired oligonucleotide at different time points to the unmodified oligonucleotide followed by MboI digestion. As the demethylation proceeded, the 35bp MboI digestion products accumulated gradually, suggesting time-dependent repair of methylated ssDNA (Fig 2E, Lanes 3-10). A significant increase of product formation was observed within the first 1h of the reaction (Fig 2F).

Having demonstrated the selectivity of our assay using pure proteins, we next wanted to determine if this could be used for high throughput screening (HTS) of AlkB inhibitors. The requirement for pure proteins often limits the use of HTS for many proteins that are difficult to express in heterologous systems. Therefore, we wanted to examine if methylated DNA repair assay could be performed using crude protein extract. Wild type and *alkB* mutant strains HK82 (Generous gift from Dr Hans Krokan, Norwegian University of Science and Technology [11]) cells were disrupted by sonication in lysis buffer (10 mM Tris–HCl, pH 8, 400 mM KCl, 2 mM EDTA, 40% (v/v) glycerol, 0.2% (v/v) triton, 2 mM DTT). After removing the cell debris by centrifugation (18000g, 30 min) supernatant was dialysed against buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 12 mM MgCl2, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT) and analyzed by SDS-PAGE (Fig. 2G, lanes 2 and 3). To carry out repair, 6.5μg of MMS-treated single-stranded DNA was incubated with 5μg of wild type and *alkB* mutant cell extract in presence of repair buffer (25μl) at 30°C for 4h. MboI digestion of repaired DNA was carried out as before. As shown in Fig. 2H, wild type cell extract efficiently repaired methylated DNA and resulted 35bp fragment release (lane 3). As

expected, MMS-treated 70bp methylated DNA was not cleaved by *alkB* mutant cell extract lacking functional AlkB protein (lane 2). Taken together, these results proved that the present assay successfully detected DNA repair activity of AlkB. Given the specificity of the method described in these experiments, it may be a useful method for future studies of DNA repair function of other Fe(II)/2-oxoglutarate-dependent dioxygenases. This low-volume assay can also be adapted to HTS using crude cell extracts containing AlkB protein.

In summary, we developed a simple method for studying *in vitro* repair of methylated DNA by AlkB. This assay for DNA repair activity employed normal agarose gel electrophoresis and ethidium bromide staining excluding the requirement of coupled-enzyme assay or radioactive labeling and denaturating PAGE. In principle, this method can also be applied to any Fe(II)/2-oxoglutarate-dependent dioxygenases that are involved in DNA repair and adapted to high throughput platforms for screening of inhibitors against these enzymes.

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References:

[1] W.K. Lutz, Endogenous genotoxic agents and processes as a basis of spontaneous carcinogenesis. Mutation research 238 (1990) 287-95.

[2] P.O. Falnes, A. Klungland, and I. Alseth, Repair of methyl lesions in DNA and RNA by oxidative demethylation. Neuroscience 145 (2007) 1222-32.

[3] S.C. Trewick, T.F. Henshaw, R.P. Hausinger, T. Lindahl, and B. Sedgwick, Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. Nature 419 (2002) 174-8.

[4] T.J. Begley, and L.D. Samson, AlkB mystery solved: oxidative demethylation of N1-methyladenine and N3-methylcytosine adducts by a direct reversal mechanism. Trends in biochemical sciences 28 (2003) 2-5.

[5] M.A. Kurowski, A.S. Bhagwat, G. Papaj, and J.M. Bujnicki, Phylogenomic identification of five new human homologs of the DNA repair enzyme AlkB. BMC genomics 4 (2003) 48.

[6] T. Gerken, C.A. Girard, Y.C. Tung, C.J. Webby, V. Saudek, K.S. Hewitson, G.S. Yeo, M.A. McDonough, S. Cunliffe, L.A. McNeill, J. Galvanovskis, P. Rorsman, P. Robins, X. Prieur, A.P. Coll, M. Ma, Z. Jovanovic, I.S. Farooqi, B. Sedgwick, I. Barroso, T. Lindahl, C.P. Ponting, F.M. Ashcroft, S. O'Rahilly, and C.J. Schofield, The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science 318 (2007) 1469-72.

[7] T.W. Roy, and A.S. Bhagwat, Kinetic studies of Escherichia coli AlkB using a new fluorescence-based assay for DNA demethylation. Nucleic acids research Vol. 35 (2007) e147.

[8] B. Sedgwick, P. Robins, and T. Lindahl, Direct removal of alkylation damage from DNA by AlkB and related DNA dioxygenases. Methods in enzymology 408 (2006) 108-20.

[9] R.S. Wu, S. Hurst-Calderone, and K.W. Kohn, Measurement of O6-alkylguanine-DNA alkyltransferase activity in human cells and tumor tissues by restriction endonuclease inhibition. Cancer research 47 (1987) 6229-35.

[10] J. Ringvoll, L.M. Nordstrand, C.B. Vagbo, V. Talstad, K. Reite, P.A. Aas, K.H. Lauritzen, N.B. Liabakk, A. Bjork, R.W. Doughty, P.O. Falnes, H.E. Krokan, and A.

Klungland, Repair deficient mice reveal mABH2 as the primary oxidative demethylase for repairing 1meA and 3meC lesions in DNA. The EMBO journal 25 (2006) 2189-98.

[11] M.P. Westbye, E. Feyzi, P.A. Aas, C.B. Vagbo, V.A. Talstad, B. Kavli, L. Hagen, O. Sundheim, M. Akbari, N.B. Liabakk, G. Slupphaug, M. Otterlei, and H.E. Krokan, Human AlkB homolog 1 is a mitochondrial protein that demethylates 3-methylcytosine in DNA and RNA. The Journal of biological chemistry 283 (2008) 25046-56.

[12] B. Sedgwick, Repairing DNA-methylation damage. Nature reviews. Molecular cell biology 5 (2004) 148-57.

[13] P. Brookes, and P.D. Lawley, Evidence for the Action of Alkylating Agents on Deoxyribonucleic Acid. Experimental cell research 24 (1963) SUPPL9:521-4.

[14] T. Lindahl, and A. Andersson, Rate of chain breakage at apurinic sites in double-stranded deoxyribonucleic acid. Biochemistry 11 (1972) 3618-23.

[15] J. Goedhart, and T.W. Gadella, Jr., Analysis of oligonucleotide annealing by electrophoresis in agarose gels using sodium borate conductive medium. Analytical biochemistry 343 (2005) 186-7.

[16] E.L. Spence, M. Kawamukai, J. Sanvoisin, H. Braven, and T.D. Bugg, Catechol dioxygenases from Escherichia coli (MhpB) and Alcaligenes eutrophus (MpcI): sequence analysis and biochemical properties of a third family of extradiol dioxygenases. Journal of bacteriology 178 (1996) 5249-56.

[17] E. Eichhorn, J.R. van der Ploeg, M.A. Kertesz, and T. Leisinger, Characterization of alpha-ketoglutarate-dependent taurine dioxygenase from Escherichia coli. The Journal of biological chemistry 272 (1997) 23031-6.

Figure Legends

Figure 1.

Preparation of DNA substrate for AlkB activity assay. (A) 70bp oligonucleotide comprised of recognition site for the restriction enzyme MboI (5'-GATC-3') in the middle. MboI digestion resulted two 35bp fragments (**B**) MMS treatment of the single-stranded DNA (ssDNA) resulted 7-meG (polygon), 1-meA (star) and 3-meC (circle) lesions. This methylated oligonucleotide was then annealed to the unmethylated oligonucleotide to produce double-stranded methylated DNA. Methylation sensitive restriction enzyme MboI could not cleave this DNA. **(C)** Agarose gel electrophoresis to resolve oligonucleotides. ssDNA and dsDNA were separated on a 3% Agarose gel using 10 mM Sodium Borate buffer and detected by ethidium bromide staining. ssDNA showed faster mobility than dsDNA. **(D)** MboI restriction digestion analysis of methylated and unmethylated oligonucleotides. Methylated and unmethylated DNA substrates were digested with MboI and analyzed by agarose gel analysis as before. Complete MboI digestion of normal substrate produced 35bp band whereas MboI did not cleave MMS-treated methylated substrate and 70bp substrate remained intact.

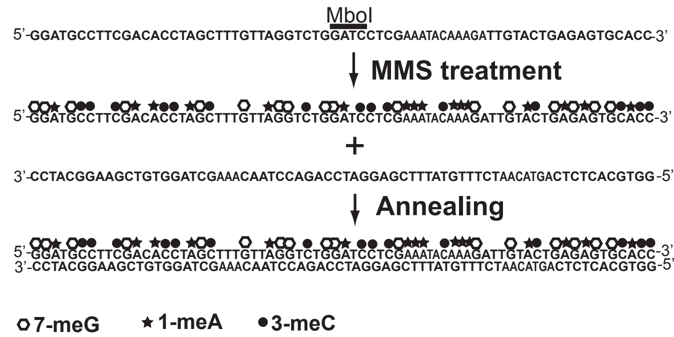
Figure 2.

Agarose gel analysis of DNA repair by AlkB. (A) SDS-PAGE analysis of recombinant proteins. GST-fusion proteins were expressed in *E.coli* cells and purified using glutathione sepharose. Proteins were visualized by staining with Coomassie blue. **(B)** Repair of methylated DNA by purified proteins. MMS-damaged DNA oligonucleotides were incubated with GST (negative control), recombinant AlkB, and two other dioxygenases including MhpB and TauD. Repair was analyzed by annealing complimentary undamaged oligonucleotide followed by MboI restriction digestion and agarose gel electrophoresis as before. The arrows indicate the position of the 70bp unrepaired substrate and 35bp band

arising due to MboI digestion of the repaired substrate. **(C)** Increasing concentration of AlkB resulted higher amount of repaired DNA. MMS-damaged DNA oligonucleotides were incubated with AlkB (0.4-2.4 μM) for 4h, and subsequently annealed to complimentary undamaged oligonucleotide and cleaved with MboI restriction enzyme. Reaction products were analyzed as before. **(D)** Intensities of the 35bp bands generated by MboI digestion of the repaired DNA were quantified by image analysis software and plotted. **(E)** Time-course of repair by AlkB. MMS-damaged DNA oligonucleotides were incubated with AlkB (0.2 μM) for 4h. Reaction products were recovered at every 0.5h interval and annealed to the complimentary undamaged oligonucleotide and cleaved with MboI restriction enzyme and analyzed as before. The arrows indicate the position of the 70bp unrepaired substrate and 35bp band arising due to MboI digestion of the repaired substrate. **(F)** Intensities of the 35bp fragment arising due to MboI digestion were quantified and plotted. Error bars represent the standard error of the mean (SEM) values. **(G)** SDS-PAGE analysis of wild type and *alkB* mutant *E. coli* crude cell extract. **(H)** Repair of methylated DNA by purified proteins. MMS-damaged DNA oligonucleotides were incubated with wild type and *alkB* mutant cell extracts. DNA repair was analyzed as before by MboI restriction digestion and agarose gel electrophoresis. Appearance of 35bp band indicates repair of methylated DNA.

A

B



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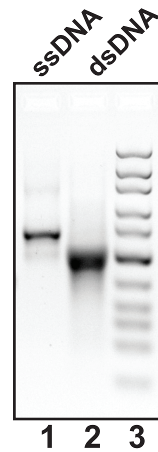

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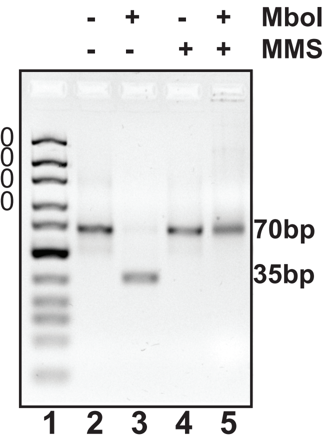

Figure 1

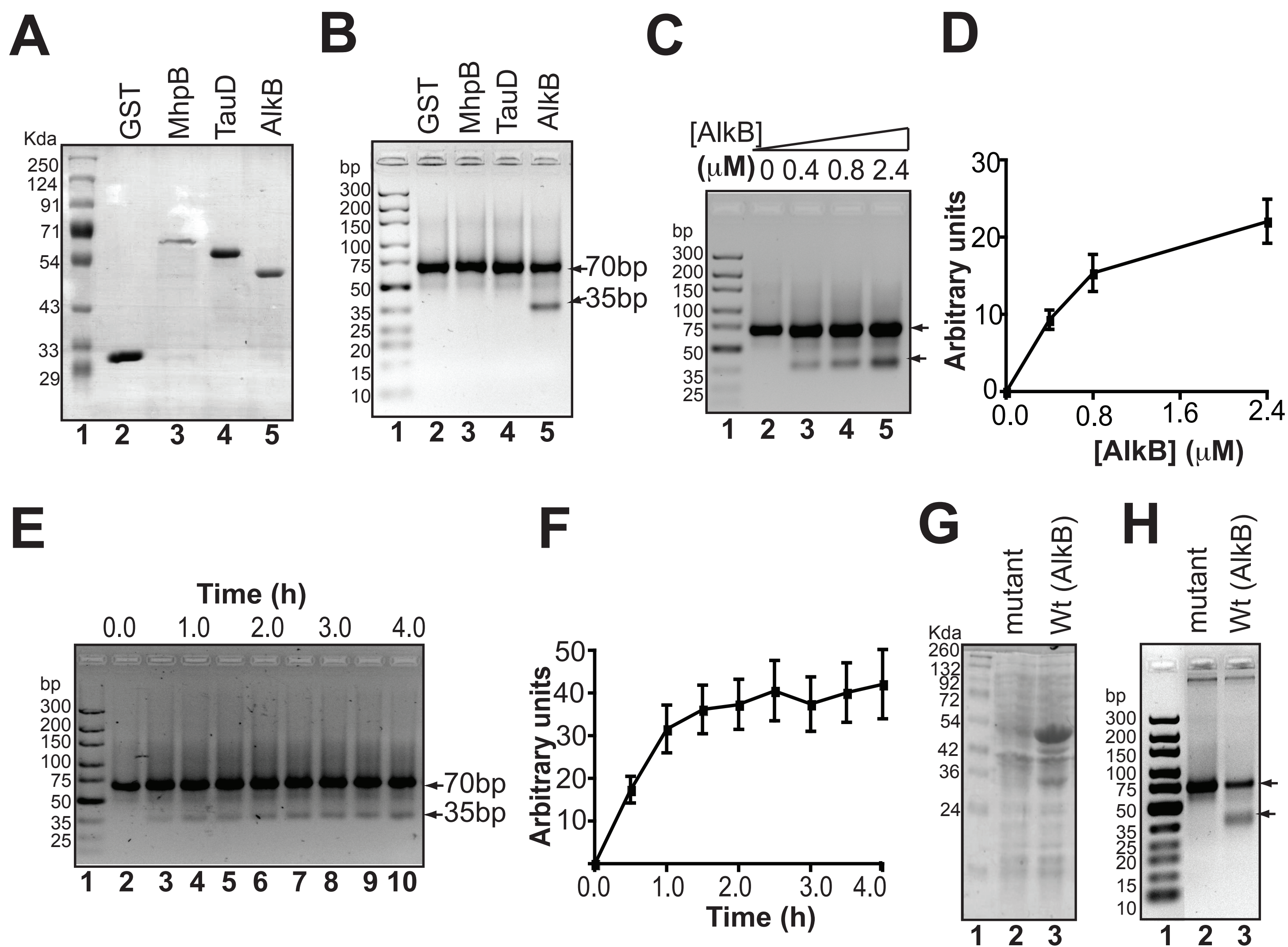

Figure 2