Published in final edited form as:

Protein Expr Purif. 2015 January ; 105: 47–53. doi:10.1016/j.pep.2014.10.005.

Expression and *in vitro* functional analyses of recombinant Gam1 protein

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Abstract

Gam1, an early gene product of an avian adenovirus, is essential for viral replication. Gam1 is the first viral protein found to globally inhibit cellular SUMOylation, a critical posttranslational modification that alters the function and cellular localization of proteins. The interaction details at the interface between Gam1 and its cellular targets remain unclear due to the lack of structural information. Although Gam1 has been previously characterized, the purity of the protein was not suitable for structural investigations. In the present study, the gene of Gam1 was cloned and expressed in various bacterial expression systems to obtain pure and soluble recombinant Gam1 protein for *in vitro* functional and structural studies. While Gam1 was insoluble in most expression systems tested, it became soluble when it was expressed as a fusion protein with trigger factor (TF), a ribosome associated bacterial chaperone, under the control of a cold shock promoter. Careful optimization indicates that both low temperature induction and the chaperone function of TF play critical roles in increasing Gam1 solubility. Soluble Gam1 was purified to homogeneity through sequential chromatography techniques. Monomeric Gam1 was obtained via size exclusion chromatography and analyzed by dynamic light scattering. The SUMOylation inhibitory function of the purified Gam1 was confirmed in an *in vitro* assay. These results have built the foundation for further structural investigations that will broaden our understanding of Gam1's roles in viral replication.

Keywords

Gam1; SUMOylation; Protein expression; Trigger factor; Cold temperature

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Introduction

Gam1, an early gene product of adenovirus CELO with a molecular weight of 31 kDa, was discovered upon screening for viral proteins that regulate cellular apoptosis [1]. Further evidence showed that Gam1 has the unique function of modulating small ubiquitin-related modifier (SUMOylation)² [2–4], a reversible posttranslational modification that alters protein functions. Many proteins are post-translationally modified to fully attain their physiological functions [5,6]. Moreover, some of these modifications, such as SUMOylation, are reversible, and confer the ability to switch protein functionality by creating different biological isoforms [7–9]. Proteins found in subnuclear compartments known as promyelocytic leukemia (PML) nuclear bodies are heavily SUMO modified, and have been postulated to play a role against viral infections [10–12]. Gam1 enhances viral replication most likely via interference with host SUMOylation and disrupting SUMOylated PML [2,4].

SUMOylation occurs when the SUMO tag is covalently linked to a lysine residue in the target protein with an isopeptide bond. Similar to ubiquitination, SUMOylation is catalyzed by an enzymatic cascade containing an E1 activating enzyme, an E2 conjugating enzyme, and a still growing number of E3 ligases. Gam1 expression diminishes global nuclear SUMOylation [3,4] by degradation of the SUMO E1 heterodimer (SAE1/2) [2] through ubiquitin ligase complexes [13,14]. It is worth noting that viruses exploit these cellular degradation pathways to manipulate normal cell processes—such as SUMOylation [12]. Gam1 was the first viral protein shown to degrade SUMO E1 and E2 enzymes [2].

To date, the structure of Gam1 is not available and the atomic details regarding the interface between Gam1 and SAE1/2, the first enzyme in SUMOylation, remain elusive. Structural investigation of the interface between Gam1 and SAE1/2 will fill the knowledge gap. Obtaining large amounts of soluble Gam1 with high homogeneity is essential to carry out the structural studies. Previous studies have produced recombinant Gam1 suitable for functional characterization [15]. However, the quantity and purity were not suitable for structural studies. In this study, we showed that a cold-shock promoter-induced, chaperone-assisted expression system (pCold-TF) significantly increases the yield and solubility of Gam1. The purifications were optimized to obtain high-purity monomeric TF-Gam1. *In vitro* SUMOylation assays indicated that recombinant TF-Gam1 is functional in inhibiting SUMOylation. These results, and the ability to produce large amounts of functional TF-Gam1, will pave the way for future structural investigations that can deepen our understanding about how Gam1 interacts with SAE1/2.

Materials and methods

Expression constructs

Seven expression constructs were generated to express Gam1 (Table 1). The two pDEST42 (N-pDEST42 and C-pDEST42) were generated using Invitrogen Gateway[®] system by

²Abbreviations used: TF, trigger factor; SUMOylation, small ubiquitin-related modifier; PML, promyelocytic leukemia; SAE1/2, SUMO E1 heterodimer; SEC, size exclusion chromatography; IEX, ion exchange chromatography; MW, molecular weight; DLS, dynamic light scattering.

sequence-specific directional DNA recombination using bacteriophage lambda recombinase [16]. Traditional restriction enzyme cloning methods were utilized for the pET (Novagen) and pCold (Takara) constructs. The original construct pSG9m [2] was used to obtain Gam1 gene for cloning into pDEST and pET vectors (Table 1). Later, the codons in the Gam1 gene were optimized for bacterial expression, *de novo* synthesized and ligated in pUC57 (Genscript) for cloning into pCold constructs. His tags such as N-terminal 6×, 10× His tags or C-terminal 6× His tags, were either engineered into the constructs by PCR, or acquired from the vector plasmids for affinity chromatography purification. Furthermore, some of the vectors contain fusion tags (GST and TF), which facilitate protein folding as well as increase solubility of the recombinant protein.

Small-scale expression trials

All constructs were transformed into BL21(DE3), or Rosetta 2(DE3)pLysS *Escherichia coli* (both from Novagen). Individual colonies were picked and grown in 3 mL LB media at 37 °C overnight. The overnight inoculant cultures were diluted (1/500) and upscaled to 50 mL LB media where growth continued until an OD₆₀₀ of 0.8 was reached. pET vector systems were induced with 1 mM IPTG at 30 °C for additional 4–16 h (Fig. 1A). pCold vector systems were grown for an additional hour at 16 °C, only then were they induced with 0.1–10 mM IPTG and subsequently grown for 12–24 h (Fig. 1B–D). After trial expression, cells were harvested and resuspended in lysis buffer (Tris pH 7.5, 10 mM imidazole, 300 mM NaCl, 0.02% NaN₃, 1 mM PMSF, and 1 mM DTT). Each 1 g of harvested bacteria was resuspended in 10 mL lysis buffer. Cell lysates were further processed via freeze–thaw cycles and cellular debris was removed by centrifugation at 20,000×g for 30 min. The resulting supernatants and pellets were assessed by SDS–PAGE, followed by Coomassie blue staining (Fig. 1).

Protein expression induced at low temperature

Conditions for protein expression were optimized with the pCold-TF-Gam1-His construct (Table 1), and these conditions were utilized for all other pCold expression constructs. Cultures were grown to an OD₆₀₀ of 0.6, at which point agitation was stopped and temperature was dropped to 16 °C for 1 h. The cultures were then induced with 0.1 mM IPTG and maintained at 16 °C for 14 h. The cells were harvested by centrifugation at 5250×g for 40 min at 4 °C. The pellets were resuspended in the same lysis buffer as in the small-scale expression trials. The resuspended cells were subsequently lysed with three freeze–thaw cycles and lysozyme treatment (0.2 mg/mL). Two cycles of high speed centrifugation (35,000×g at 4 °C for 40 min) were used to remove cell debris and un-lysed cells. The resulting lysates were then used for protein purification.

Purification of pCold-His-TF-Gam1, pCold-TF-Gam1-His and pCold-I-Gam1

Small-scale gravity flow affinity chromatography was used to optimize the purification of Gam1. About 5 mL Ni–NTA agarose beads (Qiagen) suspended in a gravity column were equilibrated with 25 mL of binding buffer (50 mM Tris pH 7.5, 300 mM NaCl, 10 mM imidazole, 0.02% NaN₃ and 1 mM DTT). 50 mL cell lysates were filtered with a 0.45 µm filter and loaded onto the column via gravity flow; the unbound proteins were washed out

with the binding buffer. The bound proteins were eluted by utilizing a step gradient from 20 to 250 mM imidazole by mixing the binding buffer with the elution buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 M imidazole, 0.02% NaN₃ and 1 mM DTT). Large-scale protein purification was performed using the optimal washing (125 mM imidazole) and eluting (210 mM imidazole) conditions established using an AKTA FPLC (Amersham) and a 5 mL HisTrap HP column (GE). To this end, about 90–100 mL cell lysate (equivalent to approximately 2.5 L of original culture) was filtered and loaded to the column, followed by washing with 30 mL 125 mM imidazole buffer and eluting with 15 mL 210 mM imidazole elution buffer.

Gam1 was further purified by size exclusion (SEC) and ion exchange (IEX) chromatography. Approximately, 5 mL of affinity purified Gam1 (~2–3 mg) were injected into a SEC column (HiLoad Superdex 26/60 200, GE) pre-equilibrated with (25 mM Tris pH 8.0, 150 mM NaCl, 0.02% NaN₃, and 1 mM DTT). The eluent from the SEC was loaded onto IEX (HiTrap Q HP, GE) pre-equilibrated in Buffer A (50 mM Tris pH 8.0, 100 mM NaCl, 0.02% NaN₃, and 1 mM DTT), and eluted with Buffer B (50 mM Tris pH 8.0, 1 M NaCl, 0.02% NaN₃, and 1 mM DTT) using a linear gradient. All samples were analyzed for purity on an SDS–PAGE stained by Coomassie blue. Protein concentration was measured by Bradford assay (Thermo scientific). Dynamic light scattering (DLS) was used to measure the hydrodynamic profile of purified Gam1 using a Zeta-sizer Nano S (Malvern).

***In vitro* SUMOylation assays**

In vitro SUMOylation reactions were carried out following an established protocol [17] with minor modifications. Briefly, 2 µL of ³⁵S-methionine labeled influenza virus NS1, a SUMOylation target [18–20], produced by a coupled transcription/translation system (Promega Corp., Madison, WI) was incubated with various amounts of recombinant TF-Gam1, 200 ng GST-SAE2/SAE1, 200 ng GST-UBC9, and 3.4 µg of His-SUMO1 in a final volume of 26 µL containing 50 mM Tris pH 7.4, 2.5 mM DTT, and 2 mM ATP. All incubations were performed at 37 °C for 90 min. The positive control contained all the components listed above without any Gam1. As a negative control, a mock protein expressed in the same pCold-TF vector system was used to determine whether the TF tag affected the *in vitro* SUMOylation. Another control of 100% SUMOylation abolishment was obtained by adding an affinity-purified catalytic domain of the yeast SUMO protease Ulp1 (Ulp1403–621) [21] 60 min after the initial reaction began. All reactions were stopped by the addition of 4× SDS–PAGE sample buffer (50 mM Tris pH 6.8, 10% glycerol, 4% SDS, 0.01% bromophenol blue, 2% β-mercaptoethanol). Samples were then boiled for 4 min, run on SDS–PAGE, and transferred to Immobilon-FL (0.45 m) membranes (Millipore Corp., Bedford, MA). Membranes were developed by autoradiography using a Personal Molecular ImagerTM and Quantity OneTM software (both from Bio-Rad Laboratories) or by immunoblotting using IRDye-conjugated secondary antibodies and infrared fluorescence imaging using an Odyssey CLx imaging system (both from Li-Cor Biosciences, Lincoln, NE). Statistical analyses were performed using GraphPad Prism, utilizing unpaired single tailed *t*-tests with Welch correction.

Results

Successful expression of Gam1 in pCold-TF constructs

The eukaryotic viral protein Gam1 is poorly expressed and mostly insoluble when produced in commonly used bacterial expression systems. Multiple expression trials of Gam1 in the pDEST42 and pET30b+ constructs resulted in either low or nondetectable amounts of Gam1 protein (Data not shown). In an attempt to improve Gam1 production, these constructs were tested in Rosetta 2 pLysS(DE3) cells [22] (EMD Millipore). These cells contain plasmids encoding tRNAs for rarely used bacterial codons, which have shown to facilitate heterologous protein expression [23]. However, no improvement of Gam1 expression was observed. In order to solve the problem of Gam1 expression, constructs with solubility-enhancing fusion tags were generated. These solubility tags have been proven to enhance expression of difficult proteins [24–26]. In the pET49b+-GST-Gam1 construct, Gam1 inducible expression was achieved (Fig. 1A). However, three major contaminants were repeatedly observed during protein purification (Fig. 1BA6–A10). Similar endogenous bacterial protein contaminations have been reported in other protein purification experiments [27,28]. Finally, after multiple trials, high level Gam1 expression was obtained using the pCold-TF construct. Although the pCold-His-TF-Gam1 construct yielded abundant recombinant protein (Fig. 1C), significant amounts of lower molecular weight proteins were observed after several steps of chromatographic purification. These lower molecular weight proteins were either incomplete translation products of TF-Gam1 or degraded TF-Gam1 from the C-terminus. Therefore, in order to purify full length Gam1 from affinity chromatography, the 6xHis tag at the N-terminus of TF was removed and added to the C-terminus of Gam1.

Purification of monomeric TF-Gam1

SDS–PAGE analysis demonstrated that recombinant TF-Gam1 represented approximately 40% of soluble proteins in the crude lysate prior to purification (Fig. 2A). Roughly 65% pure Gam1 was obtained from optimized affinity chromatography using a HisTrap HP column (Fig. 2B). Further purification using size exclusion chromatography (SEC) yielded a protein with a purity exceeding 80% as determined by Coomassie blue stained SDS–PAGE (Fig. 2C). SEC also revealed a profile of different Gam1 forms in the sample: aggregated, dimeric and monomeric (Fig. 2C). Complementary to SEC, DLS measurements allowed for an accurate assessment of the possible multimeric forms of recombinant Gam1 by determining the hydrodynamic size of TF-Gam1 in different SEC fractions (Fig 3). Based on SEC and DLS profiles, three factors were found to be critical to increase the yield of monomeric Gam1. Firstly, it was discovered that a slightly higher pH such as 7.7 lead to a higher proportion of aggregated TF-Gam1. Secondly, the addition of DTT to the purification buffers clearly triggered a shift towards monomeric Gam1 while the dimeric and aggregate forms were decreased (Figs. 2C and 3). Reducing environment could have decreased the formation of false intermolecular disulfide bonds that can cause dimerization or aggregation [29,30]. Finally, during the multiple trials to optimize the expression and purification of pCold-TF constructs, it was discovered that cooling to 16 °C for a prolonged time (1 h) before induction, increased the production of monomeric Gam1. Gam1 was further purified utilizing ion exchange chromatography (IEX) on a HiTrap Q HP column (GE), which

produced a protein estimated to be greater than 90% homogeneous by SDS–PAGE analysis (Fig. 2D).

Discussion

Both temperature and trigger factor (TF) assisted the folding of recombinant Gam1

One possible explanation for little or no protein expression in the pET constructs was that Gam1 may be toxic to bacteria in overexpression induced by IPTG and thus the proteins were targeted for degradation. Overexpression of the exogenous protein could also outpace the native folding process, leading to accumulation of unfolded protein as aggregates. Insolubility of protein overexpressed in *E. coli* is a common problem [31,32]. Therefore, co-expression with solubility enhancers was utilized to resolve these issues, such as GST and TF. TF is a prokaryotic ribosome associated chaperone (MW 48 kDa), which facilitates co-translational protein folding, thus, reducing the chances of forming misfolded and insoluble proteins [33,34]. As a native product from prokaryotes, TF is highly expressed in *E. coli*, which allowed for high yield of recombinant Gam1 [35]. Another benefit of using the pCold system is that the induction was carried out at cold temperatures, which has been shown to significantly improve protein folding by decreasing the rate of transcription and translation, thus providing more time for the protein to be folded [29,36]. In addition, at lower temperature, basal expression of endogenous bacterial proteins is reduced, further enhancing the overexpression of the recombinant Gam1. As previously mentioned, cooling to 16 °C before induction is crucial for increased production of monomeric Gam1 (Fig. 2C), suggesting that low temperature facilitated protein folding and increased the production of soluble TF-Gam1.

The use of expression tags, even as small as His-tags, does have drawbacks as they may potentially interfere with the conformation and biological functions of proteins [37,38]. For these reasons, Gam1 was cloned into pCold-I without the usage of TF. However, when induction trials were completed (Fig. 1D), recombinant Gam1 was only detected in the insoluble fraction. Lower temperatures have been shown to destabilize hydrophobic interactions [39]. Gam1 contains a hydrophobic region at the C-terminus, which is purported to be the location where EloB/C (components of the ubiquitination pathway) bind [13]. Without a chaperone to protect the hydrophobic region, Gam1 could potentially aggregate and go to the inclusion bodies. This demonstrates that cold temperature alone was not sufficient to yield soluble Gam1; TF has played a major role to protect the hydrophobic region in the properly folded monomeric Gam1, especially at low temperature. Removal of TF by protease digestion was unsuccessful, further supporting that TF and Gam1 interact closely, which protected the protease cleavage sites in the linker between TF and Gam 1.

Characterization of TF-Gam1

Previously, Gam1 has been demonstrated to inhibit global SUMOylation [4]; thus, we assessed whether recombinant TFGam1 maintained its biological function. Experiments here illustrated that when incubated with recombinant TF-Gam1, NS1 SUMOylation was abridged (lanes 5–8 of Fig. 4A), when compared to the positive and negative controls (lanes 1 and 3, Fig. 4A). These results are consistent with previous results demonstrating Gam1's

function to reduce SUMOylation [2]. However, SUMOylation was not completely inhibited by TF-Gam1. Based on quantified data (Fig. 4B), it was estimated that there was approximately 40% reduction of NS1 SUMOylation by TF-Gam1 compared to the respective controls. As mentioned in the previous discussion, TF plays a vital role in the folding of Gam1, probably protecting the C-terminus hydrophobic region and potentially hiding the contact region that binds to the E1 heterodimer. This might reduce the inhibitory efficiency of recombinant Gam1. Moreover, the reduction of conformational flexibility [40] introduced by TF could be another reason that Gam1 could not completely abolish SUMOylation. To a lesser extent, during the SEC purification of monomeric Gam1, the dimeric form cannot be completely separated (Fig. 2C), which is consistent with DLS results showing multiple peaks (Fig. 3C). Furthermore, it was observed that after SEC, storage of monomeric TF-Gam1 at 4 °C for a prolonged period of time gradually led to aggregation. This problem became more severe when samples were more concentrated. This suggests that a small percentage of dimer and aggregate fractions could also account for the reduction of TF-Gam1's functionality.

Conclusion

Gam1 is the first viral protein discovered to globally inhibit host SUMOylation via the degradation of SAE1/SAE2 and E2. Previous expression of Gam1 has yielded protein suitable for functional analyses [15], however the amount and purity are not sufficient for structural investigations. In this study, we described the various trials and exploration of multiple expression systems. We showed that most of the conventional pET vector systems produced insoluble Gam1. High level expression of Gam1 was achieved from the pCold-TF vector that utilizes cold temperature induction and a fusion tag TF to assist protein folding, both proving to be essential to yield soluble Gam1. High-purity TF-Gam1 was obtained from a series of chromatographic techniques after optimizing the buffering pH, adding reducing agents and prolonging the cooling time prior to induction. The techniques described here may also be useful for other proteins that are challenging to purify in bacterial systems. Here we also showed size exclusion chromatography enabled the separation of monomeric TF-Gam1 from its other forms, which was monitored by dynamic light scattering. Not only were large quantities of recombinant Gam1 soluble, but also functional assays verified that it maintained its biological function to a certain extent. Although TF dramatically helped the expression and purification of Gam1, it might interfere with its function. Future experiments will target co-expression of Gam1 with its native cellular counterparts to stabilize the hydrophobic interface. Nevertheless, the current TF-Gam1 constructs are useful for *in vitro* functional and structural studies. These results will also establish a foundation for the future structural determination of Gam1 with or without the binding components. Investigating the interactions between Gam1 and its cellular target proteins will deepen our understanding of how Gam1 impinges on the SUMOylation pathway as well as its roles within viral replication.

Acknowledgments

G.A.A., D.H.R., Z.L.H., P.J., S.C., J.S., G.R.A., C.X. contributed to the experimental aspects. G.A.A., S.C., J.S., G.R.A., and C.X. wrote the manuscript. We thank all undergraduates for their contribution: David Acosta, Christina Alvara, Ricardo Parra, and Scott Rush. This study is supported by UTEP new faculty startup fund and NIH-NIGMS

Grant (1SC3GM109870) to C.X., AIRC (Associazione Italiana per la Ricerca sul Cancro, IG 12075) Grant to S.C., NIH-NIAID Grant (1SC1AI098976) to G.R.A., and NIH-NIGMS Grant (1SC1GM095475) to J.S. Core facilities used for the experiments are supported by Grants from the National Center for Research Resources (5G12RR08124), and the National Institute on Minority Health and Health Disparities (8G12MD007592).

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Fig. 1.

SDS-PAGE of small-scale expression trials (A) pET49b+-GST-Gam1 supernatants with and without IPTG induction; (B) pET49b+-GST-Gam1 samples from GST affinity column: crude (CR), flow through (FT), and elution fractions (A6-A10); (C) pCold-His-TF-Gam1 pellet (P) and supernatant (S) samples with and without IPTG induction; (D) pCold-I-Gam1 supernatant and pellet samples. Molecular weight (MW) markers are in the first lane of each SDS-PAGE with the corresponding size labeled on the left. Arrows on the right of each panel point to the expected size of recombinant Gam1.

Fig. 2. SDS-PAGE and chromatograms of TF-Gam1 purification. (A) Gravity flow affinity chromatography of TF-Gam1 using gradient concentrations (40–200 mM) of imidazole to determine the optimized wash and elution conditions, FT stands for flow through; (B) TF-Gam1 affinity chromatography using GE HisTrap HP with optimized wash and elution conditions determined in (A) and labeled on the chromatogram; (C) size exclusion chromatography (SEC) purification of TF-Gam1 after (B). Left (without DTT) and right (with DTT) panel showing chromatograms before and after optimized purification conditions, respectively. Three peaks of aggregation (AG), dimeric (DM) and monomeric (MO) forms of TF-Gam1 are depicted by arrows; (D) ion exchange chromatography (IEX) purification of TF-Gam1 after (C). Lanes of SDS-PAGEs of TF-Gam1 are shown in panel C and D with arrows pointing to target peaks.

Fig. 3. The hydrodynamic size profile of TF-Gam1 characterized by dynamic light scattering (DLS). (A), (B), and (C) represent the aggregated, dimeric, and monomeric forms of TF-Gam1 eluted from SEC, respectively. DLS profiles can be used to determine TF-Gam1 forms.

Fig. 4.

TF-Gam1 decreases the *in vitro* SUMOylation of NS1. (A) *In vitro* translated NS1 (a well characterized SUMO substrate [18–20]) was incubated in the presence of a reconstituted SUMOylation mix comprising purified GST-SAE2/SAE1, GST-UBC9 and an ATP-containing buffer, with or without increasing amounts of TF-Gam1 for 90 min at 37 °C. Reactions were stopped as described in Materials and Methods, resolved by SDS–PAGE, and scanned using autoradiography. Arrows on the left point to the expected size of SUMOylated/Un-SUMOylated substrate. (B) The *in vitro* SUMOylation efficiency was quantified by densitometric scans of (A) for the total amount of SUMO modified substrate using the un-SUMOylated substrate as internal standards. Three replicates were performed to estimate the standard errors. Statistics *t*-test was applied and *p* values were calculated and labeled accordingly above the bar graphs. Signifies statistical significance using conventionally accepted *p* < 0.05 criteria. Samples are labeled below each lane in panel (A) and each corresponding bar in panel (B). For each protein sample tested, a “low” amount (1.2 µg) and a “high” amount (2.4 µg) were used in the reaction to show the dose dependent inhibitory effects of recombinant Gam1 on SUMOylation.

Table 1

Constructs and primer sequences.

Construct	Primers
pDEST42-His-Gam1 (the PCR was done in three steps)	Sense #1 (step one)
	5'-GCGGCTATTGAAGGTCGCGCCCGCAACCCATTCCGC-3'
	Sense #2 (step two)
	5'-CACCACCACCACCACCACCACCCTCCGCGGCTATTGAAGGTCGC-3'
	Sense #3 (step three)
	5'- <u>CACCATGGCAGCAGCACACCACCACCACCACCACCAC</u> -3'
	CACC overhang for Invitrogen Gateway® cloning
	Antisense:
	5'- <u>TTACAGAGAATGGTAGGGGTGGTGCCGAGACCCGAGTCC</u> -3'
	Stop codon
pDEST42-Gam1-His	Sense:
	5'- <u>CACCATGGCCCGCAACCCATTCCGCATGTTCCCTG</u> -3'
	CACC overhang for Invitrogen Gateway® cloning
	Antisense:
	5'-AGCTGCGGAGCGACCTTCAATCAGAGAATGGTAGGGGTGGTGC-3'
pET30b+-Gam1-His	Sense:
	5'- <u>CGCCCATATGGCCCGCAACCCATTTCGCATGTTCCCT</u> -3'
	NdeI
	Antisense:
	5'- <u>CGCGCTCGAGATGATGATGATGCAGAGAATGGTAGGGGTG</u> -3'
	XhoI
pET49b+-GST-Gam1 (the PCR was done in two steps)	Sense #1 (step one)
	5'-GGTAGTATTGAAGGTCGCGCCCGCAACCCATTCCGC-3'
	Sense #2 (step two)
	5'-GCGC <u>ACTAGTGGTGGTAGTATTGAAGGTCGCGCC</u> -3'
	SpeI
	Antisense:
	5'-GCGC <u>CTCGAGTTACAGAGAATGGTAGGGGTGGTG</u> -3'
	XhoI
pCold-His-TF-Gam1	<i>De novo</i> synthesized construct (Genscript) in pUC57 digested with 5'-NdeI and 3' HindIII
pCold-I-Gam1	<i>De novo</i> synthesized construct (Genscript) in pUC57 digested with 5'-NdeI and 3' HindIII
pCold-TF-Gam1-His	Sense:
	5'-GCGCTAC <u>ATATGGCTCGTAATCCGTTTCGTATGTTCCGGGCGACCTGCCGTACTACATGG</u> -3'
	NdeI
	Antisense:
	5'-GGT <u>CGAATTC</u> TTAATGATGATGATGATGATGGCGACCTTCAATCAGCGAATGATACGGATGAT-3'
	EcoRI