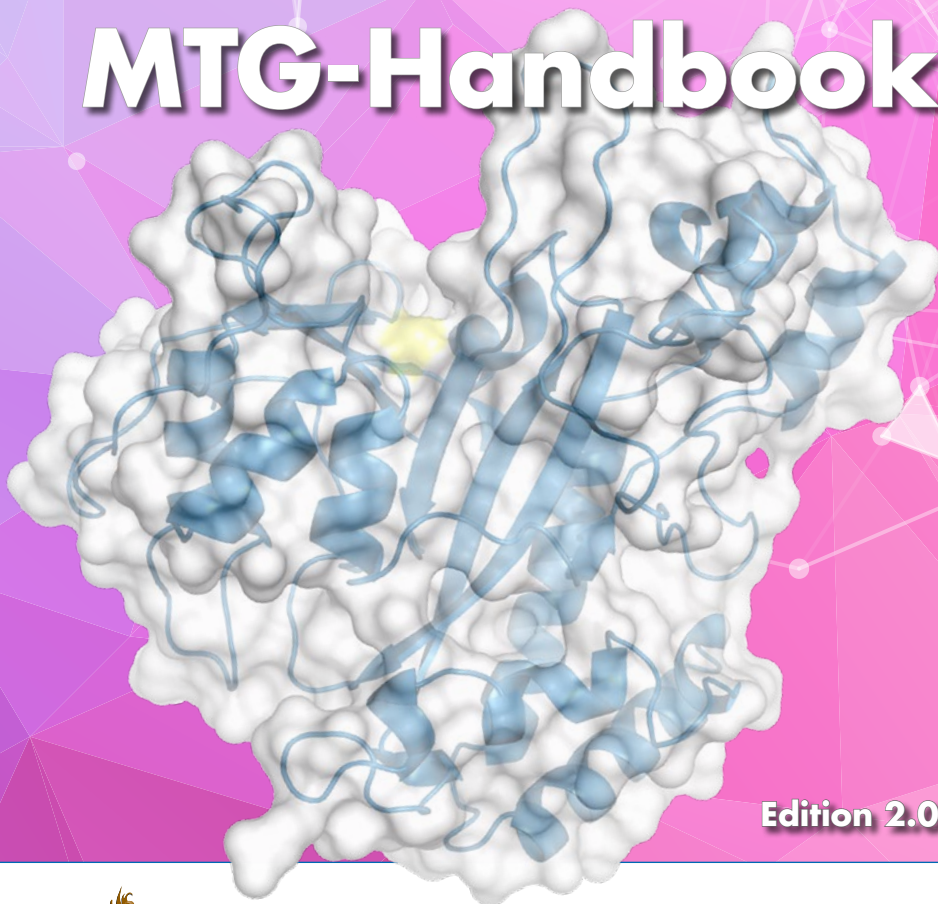


**Zedira`s**

# **MTG-Handbook**



**Edition 2.0**



*Follow me on a journey on MTG!*



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Microbial transglutaminase (MTG) catalyzes cross-linking of proteins. In simple words: MTG is a biological glue. MTG is of major importance to industry, especially in the food sector, due to its protein cross-linking activity.

Moreover, MTG not only can cross-link proteins, but it can also incorporate primary amines into proteins in a covalent manner. This feature allows the enzyme to link a large variety of compounds to proteins, including biotin, fluorescent dyes, click chemistry substrates, and cytotoxins.

Zedira has a long history regarding MTG. It was in the late 1990s, when Ralf – working toward his PhD thesis in Prof. Hans-Lothar Fuchsbauer's lab - discovered that MTG is secreted by *Streptomyces mobaraensis* as a proenzyme. After having worked for more than 30 years on MTG, Hans-Lothar recently, authored an impressive and highly recommendable review on MTG: Approaching transglutaminase from *Streptomyces* bacteria over three decades (FEBS J, 2021, June 8).

The scientific findings of the Fuchsbauer-lab have become translated into business. N-Zyme BioTec founded in 1999, followed by Zedira in 2007, had and still has the mission to provide reliable tools and technologies to transglutaminase researchers worldwide. Today, Zedira's portfolio of transglutaminase specialty reagents encompasses more than 300 products.

Recombinant MTG – one of our very first products (T001) – has attracted more and more interest, resulting from its unique ability to generate site specific and homogeneous antibody drug conjugates (ADCs).

Zedira – as the producer of high-quality recombinant MTG – promises to fulfill to the best of our ability our customers' requirements, not only in quality and quantity, but also by meeting stringent regulatory requirements: In 2019 Zedira was awarded an ISO9001:2015 certification. Now, we introduce **Andracon™** (T153) the novel trademark product of ultra-pure and highly active MTG for ADC production and other sophisticated applications.

In order to provide an overview of this fascinating enzyme, we published Zedira's MTG-Handbook in February 2020. Following our customers' much appreciated feedback, this 2<sup>nd</sup> edition of Zedira's MTG-Handbook includes additional information on MTG's characteristics that are useful for everyday work in the lab: pH- and temperature profiles, stability and more. In addition, a starting protocol for antibody conjugation is included.

We highly appreciate the blogs of renowned colleagues from the MTG community whose work and suggestions have been of great help in putting together the current handbook.

## Zedira's Microbial Transglutaminase – The Standard in ADC-Production

### 👉 Unrivalled quality and support!

	MTG (R&D)	Andracon™
	T001	T153
• Recombinantly produced in <i>E. coli</i>	✓	✓
• Ultra-pure and highly active	✓	✓
• Batch to batch consistent quality	✓	✓
• Scientific and technical support	✓	✓
• Available from 25 U vials up to bulk amounts	✓	✓
• License free	✓	✓
• Produced according to an SOP in an ISO9001:2015 certified environment	✓	✓
• Production grade MTG (e.g. production of antibody drug conjugates)		✓
• Characterized for host cell protein, host cell DNA, and endotoxin-levels		✓
• Customer audits for qualification according to guideline ICH Q7 possible		✓

### Questions?

Customer-specified filling or formulation required?

Please contact us for: [MTG@zedira.com](mailto:MTG@zedira.com)

We are looking forward to talking to you!

On behalf of the entire team at Zedira

Martin Hils and Ralf Pasternack



# 1 Transglutaminases

The history of the enzyme class of transglutaminases started more than 60 years ago, when the term transglutaminase (TG) was introduced in scientific literature by Heinrich Waelsch and co-workers.

Transglutaminases are defined as R-glutaminy-peptide: amine  $\gamma$ -glutamyl-transferase (EC 2.3.2.13). They use a modified double-displacement mechanism to carry out an acyl transfer reaction between the  $\gamma$ -carboxamide group of a peptide-bound glutamine residue and the  $\epsilon$ -amino group of a peptide-bound lysine (Figure 1). The active site in mammalian transglutaminases consists of a catalytic triad (Cys, His and Asp). The active site cysteine reacts with the  $\gamma$ -carboxamide of the glutamine, forming a  $\gamma$ -glutamyl thioester resulting in the release of ammonia. This activated species subsequently reacts with nucleophilic primary amines, yielding either an isopeptide bond (pathway ①) or a ( $\gamma$ -glutamyl)amide bond (pathway ②). When an amine is not available, the acyl-enzyme intermediate can be hydrolyzed to yield glutamic acid (pathway ③).

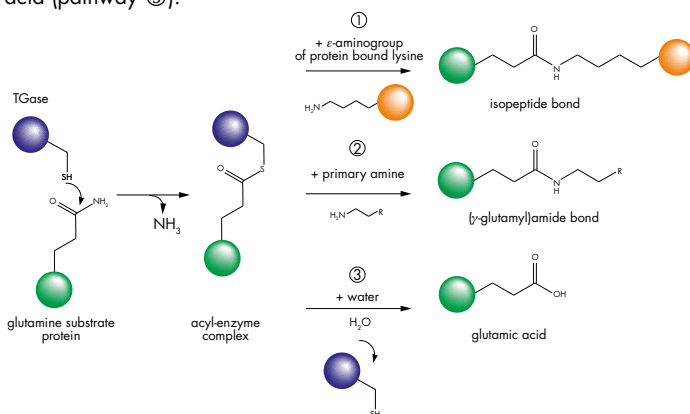


Figure 1: Reaction pathways of transglutaminase.

Today, we know that transglutaminases are produced by species of all taxonomic domains. Transglutaminases were described from microorganisms, plants, invertebrates, fish, amphibians, reptiles, birds and mammals (for review see Gerrard et al. 2001, Griffin et al. 2002). In humans, even nine transglutaminase genes are present, eight coding for active transglutaminases, one for an inactive membrane protein (Griffin et al. 2002). Important to mention, eukaryotic transglutaminases are not related to microbial transglutaminases, but the result of convergent evolution. Essentially, eukaryotic transglutaminases require Ca<sup>2+</sup> ions as cofactor, while microbial transglutaminases catalyzes the cross-linking reaction without cofactor requirement.

## 2 Microbial Transglutaminase – Structure

In the late 1980ies the Japanese companies Amano Enzyme® and Ajinomoto® screened 5,000 microorganisms seeking a cheap and stable transglutaminase for food applications. *Streptovercillium* sp. S-8112, later on determined as *Streptovercillium mobaraense* and reclassified as *Streptomyces mobaraensis* was found to produce a  $\text{Ca}^{2+}$  independent and stable transglutaminase, named microbial transglutaminase (MTG or synonymous BTG for bacterial transglutaminase) (Ando et al. 1989; Washizu et al. 1994; Witt and Stackebrandt 1990).

Since then, MTG is produced in an industrial scale and marketed by Ajinomoto® under the brand Activa®. Upon patent expiry additional producers entered the market. Based on different formulations, the enzyme is widely used to modulate the texture and properties of protein containing food. Also, innovative non-food applications using protein cross-linking have been described.

The MTG-gene is translated into a prepro-enzyme consisting of 407 amino acid residues. The hydrophobic 31 amino acid long pre-peptide is required for efficient protein secretion. It is followed by the pro-peptide and the catalytic transglutaminase domain. In case of *Streptomyces mobaraensis* the pro-peptide consists of 45 amino acids rendering the enzyme inactive [Pro-enzyme [zymogen], 42 kDa]. The pro-peptide protects the strain from uncontrolled cross-linking reactions. The extracellular pro-enzyme is activated by cleavage of the N-terminal pro-peptide (4.6 kDa). The mature enzyme has a mass of 38 kDa (Pasternack et al. 1998; Washizu et al. 1994). The primary structure is given in Figure 2.

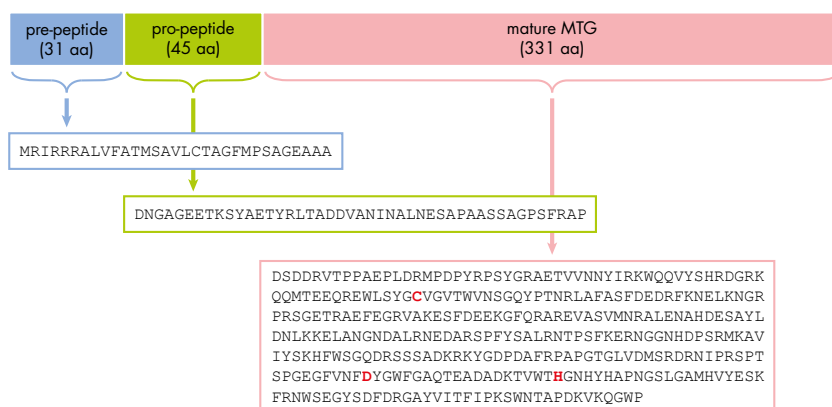


Figure 2: Primary structure of microbial transglutaminase prepro-enzyme. Proposed catalytic triade consisting of amino acids cysteine, aspartate and histidine is marked in red. Figure adapted from Kanaji et al. 1993; Kashiwagi et al. 2002; Pasternack et al. 1998.

In *Streptomyces mobaraensis* MTG is activated in two steps. First, the pro-peptide is cleaved by transglutaminase-activating metalloproteinase (TAMP), yielding an already active MTG with the N-terminus FRAPDSDDR... . In the consecutive step, the tetrapeptide FRAP is cleaved by tripeptidyl aminopeptidase (TAP), resulting in the fully processed MTG with the DSDDR... N-terminus [Zotzel et al. 2003a; Zotzel et al. 2003b]. The processing of MTG is shown in Figure 3.

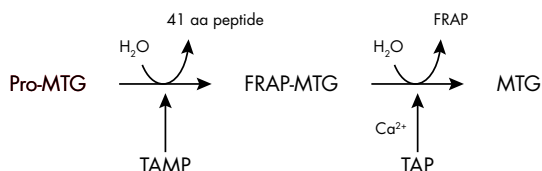


Figure 3: Processing of Pro-MTG from *S. mobaraensis* by proteases TAMP and TAP. After the transglutaminase-activating metalloproteinase (TAMP) removed most of the pro-peptide, resulting in an already active MTG, the FRAP tetrapeptide is cleaved by the tripeptidyl aminopeptidase (TAP) to yield the mature MTG. Figure adapted from Zotzel et al. 2003b.

Recombinantly produced Pro-MTG can be processed by several proteases, like Dispase® from *Bacillus polymyxa*, chymotrypsin, trypsin [Pasternack et al. 1998; Pfeiderer et al. 2005] as well as proteinase K, cathepsin B and thrombin (see Figure 4) [Marx et al. 2008].

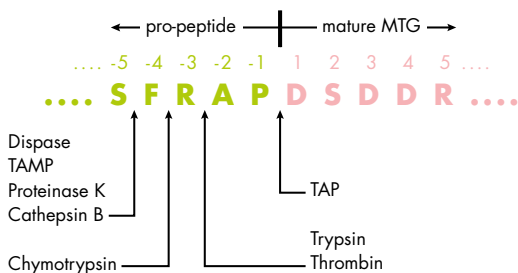


Figure 4: Amino acid sequence in the proteolytic cleavage area between pro-peptide and mature MTG from *S. mobaraensis*. Specific processing sites of proteases are marked with arrows. Figure adapted from Zotzel et al. 2003a.

The crystal structure of MTG has been determined by Kashiwagi and co-workers at 2.4 Å. MTG belongs to the  $\alpha$ + $\beta$  folding class. It consists of 11  $\alpha$ -helices and 8  $\beta$ -strands [Kashiwagi et al. 2002].



The protein folds into a disc like shape with a deep cleft on the edge of the molecule (Figure 5). The residue Cys64 is located at the bottom of this cleft, named active site cleft, which is essential for catalytic activity of MTG. The residues Asp255 and His274, also important for the catalytic mechanism (see chapter 4), are in proximity of Cys64 (Figure 6B).

The model suggests exposure of Cys64 to the solvent. Thus, it can react promptly with substrates. Furthermore, the flexibility of the right-side wall of the active site cleft minders steric hindrance between enzyme and substrates.

At the entrance of the active site cleft resides the N-terminus. The Pro-MTG structure deposited in 2009 by Yang et al. at the protein data bank PDB revealed that the pro-peptide covers the active site cleft, rendering the zymogen inactive (Figure 5A).

MTGs three-dimensional structure is unique and completely different from human transglutaminases' structures.

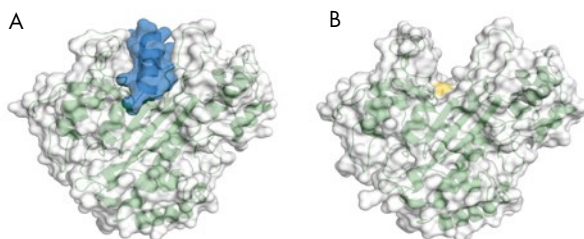


Figure 5: Microbial transglutaminase crystal structures. A) Pro-Transglutaminase (PDB-ID: 3IU0). The pro-peptide is shown in blue. B) active Transglutaminase (PDB-ID: 1IU4). Active site cysteine is marked in yellow.

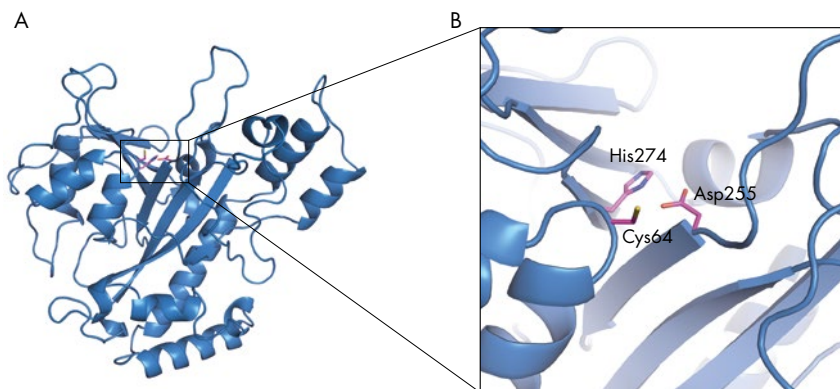


Figure 6: Ribbon model of microbial transglutaminase from *S. mobaraensis*. A) Tertiary structure with highlighted proposed catalytic triade in the active side cleft. B) Enlarged active side with residues Cys64, Asp255 and His274 (PDB code 1IU4).

### 3 Characterization of recombinant MTG

Recombinant microbial transglutaminase (Art. No. T001 and T153) is produced in *E. coli* with the gene derived from *Streptomyces mobaraensis*. Table 1 summarizes biochemical and biophysical parameters of recombinant FRAP-MTG compared to wild type MTG produced in *Streptomyces* strain. In the following, characterization of recombinant MTG is presented in more detail.

Table 1: Summary on MTG.

Parameter	Wild type MTG	Recombinant FRAP-MTG
N-terminus	DSDDR...	FRAPDSDDR...
Amino acids	331	335
Size (kDa) <sup>1</sup>	37,861	38,333
Charge (calc) <sup>2</sup>	+0.25	+1.25
pI (calc) <sup>2</sup>	7.09	7.45
pI	8.9 <sup>3</sup>	n.d.
Temperature optimum	50°C <sup>3,4</sup>	55°C <sup>4,6</sup>
pH-range	5 – 9 <sup>3,5</sup>	5 – 9 <sup>5,6</sup>
pH-optimum	6 – 7 <sup>3,5</sup>	5 – 8 <sup>5,6</sup>

<sup>1</sup> Mass spectrometry data

<sup>2</sup> DNASTAR Editseq

<sup>3</sup> Ando et al. 1989

<sup>4</sup> at pH 6

<sup>5</sup> at 37°C

<sup>6</sup> Zedira data

#### Transamidation and Deamidation

As shown in Figure 1, section 1, the first step of the transglutaminase reaction results in a thioester bond between transglutaminase's active site cysteine and the (already deamidated) substrate glutamine. This thioester bond can be resolved by reaction with a primary amine (transamidation) or a water molecule (deamidation).

MTG's transamidation and deamidation activities were analyzed based on a gliadin-derived peptide (PepQ) in the presence (transamidation) or absence (deamidation) of lysine as primary amine substrate (for detailed description see Heil et al. 2016).

Figure 7 shows the pH-dependent activity of deamidation and transamidation. The maximum activity for both reaction types is given at pH 5 (see also section pH-profile, Figure 11). Transamidation is favored over deamidation with faster reactions rates (about 12-times at pH 5 to 57-times at pH 9). Overall, transamidation is less affected by pH than deamidation. Increasing temperatures result in increased activity, but temperature does not affect the preference of transamidation over deamidation (ratio = 15 +/-1).

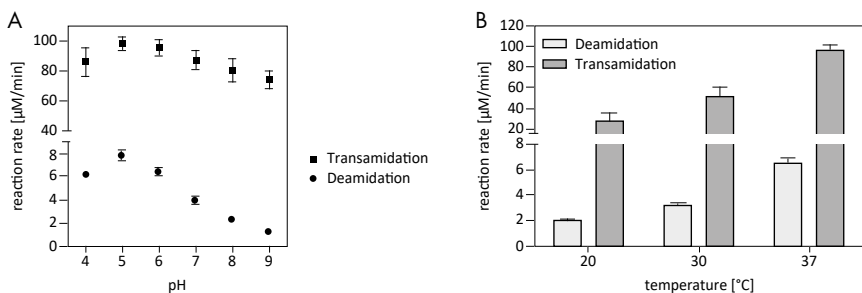


Figure 7: Deamidation and transamidation of PepQ by MTG. A: Reaction rates at different pH. B: Temperature effect on deamidation and transamidation activity. Figure adapted from Heil et al. 2016.

Deamidation was shown to depend on primary amine concentration (Figure 8). At a molar PepQ:lysine ratio of 2:1, equal amounts of deamidated and transamidated reaction products were formed. At a molar PepQ:lysine ratio of 1:2.5 no deamidation was observed anymore. Within the tested lysine concentration range, the transamidation reaction rate was linearly dose dependent accompanied by decreasing deamidation reaction rates

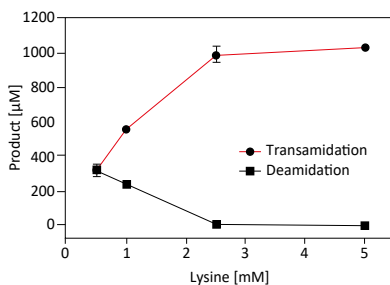


Figure 8: Effect of lysine concentration at pH 6 on reaction product formation (substrate: PepQ, 37°C, 120 min). Figures adapted from Heil et al. 2016.

## Temperature Profile

The temperature profile of MTG was determined by the incorporation of hydroxylamine into the dipeptide-derivative ZQG-OH (hydroxamate assay) as well as by an antibody conjugation approach.

The hydroxamate assay reveals an activity increase up to a maximum at 55°C. Temperatures higher than 60°C led to severe activity loss (Figure 9).

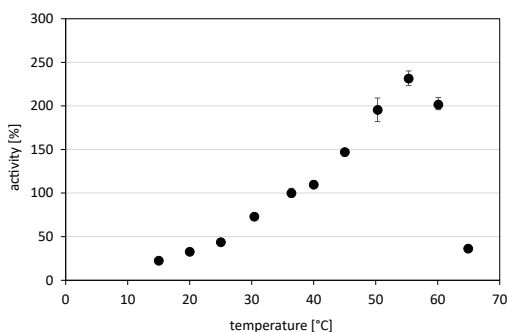


Figure 9: Temperature profile of MTG (T001). Activity was measured using hydroxamate based ZediXclusive Microbial Transglutaminase Assay Kit (Z009). Activity at 37°C was set to 100%.

Based on these results, MTG activity was determined in an antibody conjugation (labeling) setting. Boc-Lys-en-DNS (KxD, K004) was incorporated into deglycosylated human IgG in the temperature range from 34 to 55°C. Conjugation efficiency was visualized by UV-excitation of dansylated antibody in an SDS-PAGE gel (Figure 10).

Antibody staining performed well from 34 to 48°C with an optimum at 41°C. At higher temperatures (52 – 55°C) signal intensity decreases, indicating a lower labeling efficiency / antibody loss.

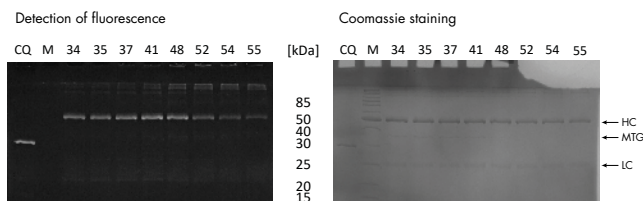


Figure 10: Temperature profile of MTG in antibody (deglycosylated IgG) labeling. Left: Detection of fluorescence upon UV excitation. Right: Coomassie stained PAGE-Gel (12.5%). CQ = Control protein fused with Q-tag (MTG-substrate sequence); M = PageRuler™ unstained Protein Ladder; HC = antibody heavy chain; LC = antibody light chain.

## pH Profile

The pH-dependence of MTGs transamidation activity was determined at pH5 (citrate buffer) and pH 6-9 (Tris-buffer) in the hydroxamate assay (hydroxylamine incorporation into ZQG-OH). MTG is highly active at pH 5-8 and starts losing activity at pH 9 (Figure 11). The moderate pH-dependence at this pH-range was also shown in the setting described in Figure 7.

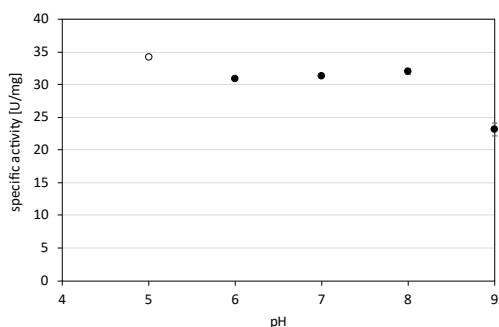


Figure 11: pH profile of MTG (T001) at 37°C. Activity was measured using the hydroxamate-assay (ZediXclusive Microbial Transglutaminase Assay Kit; Z009). Citrate buffer was used for measurements at pH 5 (open circle). Tris-buffer was used for measurements at pH 6-9 (filled circles).

Moreover, conjugation of deglycosylated human serum IgG with Boc-Lys-en-DNS (KxD, K004) (Figure 12) was performed at pH 4-9. Conjugation by MTG performed well from pH 5 – 9 with an optimum at pH 6-8.

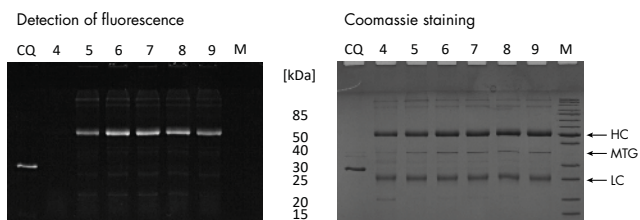


Figure 12: pH profile of MTG (T001) in antibody labeling. Left: Detection of fluorescence by UV excitation. Right: Coomassie stained PAGE-Gel (12.5%). CQ = Control protein fused with Q-tag (MTG-substrate sequence); M = PageRuler™ unstained Protein Ladder; HC = antibody heavy chain; LC = antibody light chain.

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## Stability in solution at 4°C

Liquid MTG can be stored at 4°C up to 6 days hardly losing any catalyzing activity. A longer storage period up to 40 days leads to about 20% activity loss (Figure 13).

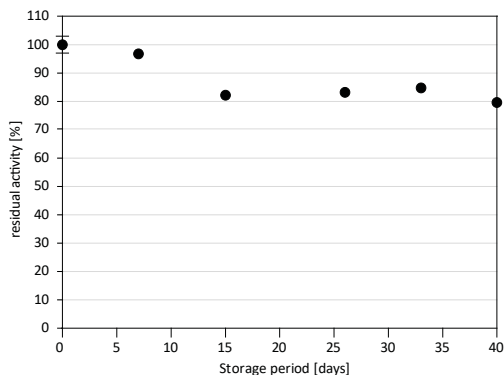


Figure 13: Stability of MTG in solution. Storage of liquid T001 at 4°C up to 40 days.

## Freeze (-80°C) – Thawing Stability

Upon reconstitution of freeze-dried MTG, storage of undiluted working aliquots preferably at -80°C is recommended. Repeated freezing and thawing is generally not recommended. However, no significant activity loss was observed within 10 freeze/thaw cycles (Figure 14).

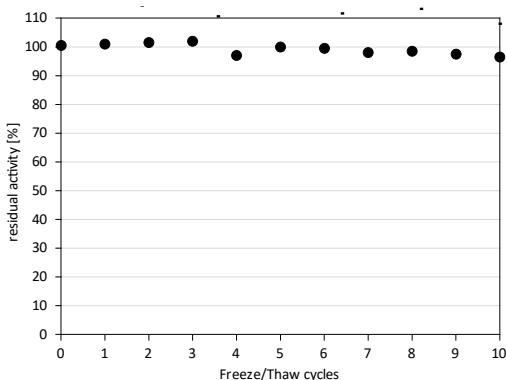


Figure 14: Stability of MTG activity over 10 freeze/thaw cycles at -80°C. Activity was determined with the hydroxamate assay (Z009).

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## Robustness to DMSO

Small molecular substrates for MTG-catalyzed conjugation are often dissolved in DMSO. MTG-activity is not affected at DMSO-concentrations up to 15% (however, high DMSO-concentrations may be detrimental to the protein to be conjugated). A small activity loss (4%) was observed for 20% DMSO (Figure 15).

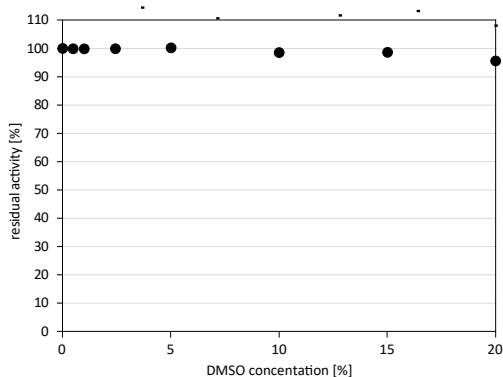


Figure 15: Robustness of MTG (T001) to increasing DMSO-concentrations. Activity was determined with the hydroxamate assay (Z009).

## 4 Biology of Microbial Transglutaminase

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Microbial transglutaminase is an enzyme essentially of industrial interest, documented by a multitude of publications on technical applications. While its production as preproenzyme and its maturation processing is well understood (see section 2), little is known about MTG's physiological role. Chen et al. describe that *Streptomyces hygroscopicus* lost the ability to form aerial hyphae upon disruption of the MTG-gene (Chen et al. 2010). Lothar Fuchsbauer's group identified several extracellular protease inhibitors and protease autolysis inducers as transglutaminase substrates.

These findings are hints that MTG might play a role in cell wall and hyphae formation of some *Streptomyces* species and may be also in the protection from proteolytic attacks by covalent incorporation of protease inhibitors (Sarafeddinov et al. 2011; Sarafeddinov et al. 2009; Schmidt et al. 2008).

Natural MTG-substrates are in the focus of Hans-Lothar Fuchsbauer's review "Approaching transglutaminase from *Streptomyces* bacteria over three decades" (FEBS J, 2021, June 8).





## 5 Enzymology

Kashiwagi et al. 2002 postulated a cysteine protease-like catalytic mechanism for microbial transglutaminase, based on the structural similarity of MTGs active site with human factor XIII, where Cys314, His373 and Asp396 form a catalytic triad.

However, in MTG, Asp255 plays the role of the His residue in human factor XIII-like transglutaminases, resulting in a catalytic dyad with Cys64 and Asp255 for the cross-linking reaction. His274 is not essential though. This was shown by His274Ala MTG mutants that still showed 50% of activity compared to wild type MTG. His274 might only be responsible for maintaining structural conformation of the active site by forming a hydrogen bond between the  $\gamma$ -carboxyl group of Asp255 and the imidazole group of His274.

In the proposed first reaction step (Figure 16A), the side chain of the substrate glutamine residue (acyl donor) is nucleophilically attacked by the thiolate ion of Cys64. As a note, it is not clear if the aspartate is able to deprotonate the cysteine to yield the thiolate. In the next steps (B and C), the initially neutral Asp255 donates a proton to the acyl-enzyme complex, while ammonia is released (and immediately protonated to ammonium). In the following, an acyl acceptor, like the side chain of protein bound lysine residue, enters the active site. Since the side chain of Asp255 is now negatively charged, it accepts a proton from the lysine (D). In the final step, the product is released from the tetrahedral oxyanion intermediate (E and F), yielding either an isopeptide bond or a ( $\gamma$ -glutamyl)amide bond.

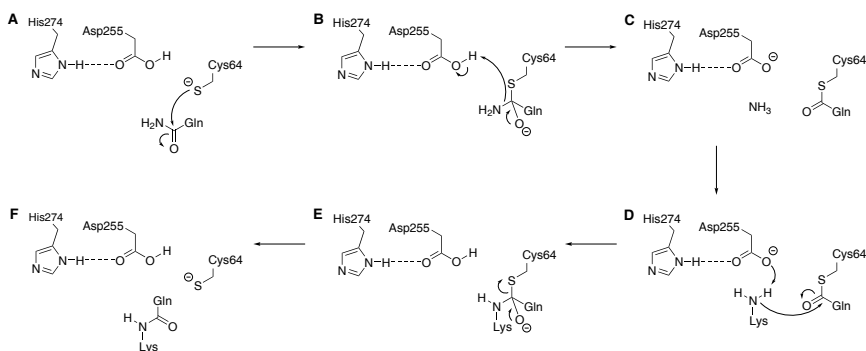


Figure 16: Hypothetical catalytic mechanism of microbial transglutaminase according to Kashiwagi et al. 2002. Glutamine and lysine are protein bound substrates.

MTG has a higher reaction rate as well as lower substrate specificity compared to eukaryotic transglutaminases (Shimba et al. 2002). Reasons for that might be the exposure of Cys274 to the solvent as well as the high flexibility of the active site cleft (Kashiwagi et al. 2002).

It was described by Matsumura and coworkers, that spherical proteins (e.g.  $\alpha$ -lactalbumin) are unsuitable as MTG substrates (Matsumura et al. 1996). However, partial disruption of proteins tertiary structure by treatment with reducing agents (e.g. DTT or  $\beta$ -mercaptoethanol) as well as increased temperature, pressure or changes in pH improved substrate properties (Ikura et al. 1984; Nonaka et al. 1997). After investigation of substrate reactivity based on proteins secondary structure, it was shown, that glutamine residues in flexible and unstructured secondary structures exhibit better MTG substrate properties (Hayashi 1997). Similar results were shown for lysine residues (Spolaore et al. 2012).

Table 2 summarizes some protein substrates for MTG.

Table 2: Substrates of microbial transglutaminase.

Modified Proteins	References
<i>S. mobaraensis</i>	
$\beta$ -Lactamase	Zindel et al. 2016
SSTI	Schmidt et al. 2008
SPI	Sarafeddinov et al. 2011
DAIP	Sarafeddinov et al. 2009
Food technology	
Serum albumin (pt)	Nonaka et al. 1989
Conalbumin (pt)	
$\alpha$ -Casein	
7S, 11S globulin	
Myosin	
Glycinin	Kang and Cho 1996
Globulin	Siu et al. 2002
Ovomucin (pt)	Kato et al. 1991
Actin (pt)	Huang et al. 1992
Antibodies	
IgG1 (deglycosylated)	Jeger et al. 2010

(pt) = pre-treated

MTG has stringent substrate specifications regarding its acyl-donor-substrate. It reacts only with L-glutamine, but not with other glutamine-analogues with varying length of the side chains (Ohtsuka et al. 2000a). The glutamines neighboring amino acids are of crucial importance (Sugimura et al. 2008). Hydrophilic as well as hydrophobic amino acids in glutamine's vicinity improve the substrate properties of a peptide or protein bound glutamine.

Sugimura et al. (2008) described the relevance of positions -3, -1, +1, -2 and +3 (glutamine: position 0) of peptide substrates. Out of  $1.5 \times 10^{11}$  random phage clones, three sequences (M5, M42, M48) with exceptionally good substrate properties were found (Figure 17). Their common features were: an aromatic amino acid at position -3; at positions -1, +1 and +2 the amino acids leucine, arginine and proline; at position +3 or +4 the amino acid tyrosine was located (Sugimura et al. 2008). Later it was shown, that these sequences can be reduced to heptamer structures (Oteng-Pabi and Keillor 2013; Oteng-Pabi et al. 2014).

Pos.:	-3	-2	-1	0	1	2	3	4	5	6	7	8
M5	W	A	L	Q	R	P	Y	T	L	T	E	S
M42	Y	E	L	Q	R	P	Y	H	S	E	L	P
M48	W	A	L	Q	R	P	H	Y	S	Y	P	D

Figure 17: MTG substrate sequences according to Sugimura et al. (2008). The red box marks the heptamer substrate sequences according to Oteng-Pabi et al. (2014).

Ohtsuka et al. (2000) described a negative influence of glycine or positively charged amino acids at the glutamine's N-terminal side in synthetic peptides as well as positive effect of glycine at the glutamine's C-terminal side on MTG substrate properties (Ohtsuka et al. 2000b).

Based on MTG's crystal structure (Kashiwagi et al. 2002) the observations of Sugimura et al. (2008) can be explained. A charged region of the active site cleft (b in Figure 18) as well as hydrophobic amino acids (a in Figure 18) bind the hydrophobic substrates N-terminus. Arginine at position +1 is bound by the acidic region of the active site cleft (Sugimura et al. 2008).

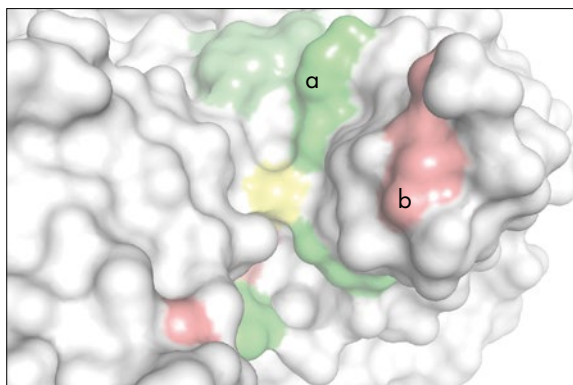


Figure 18: Active site cleft of microbial transglutaminase from *S. mobaraensis* (according to Gundersen et al. 2014). Yellow: reactive site Cys64; a: hydrophobic region of active site cleft; b: acidic region binding arginine of substrate sequence (pos. +1).

Requirements for acyl acceptors (peptide or protein bound lysine or primary amines) are broad compared to acyl donors' requirements. Next to the natural acyl acceptors, basically every amino group with different functional groups and a spacer of at least three C-atoms (or longer) can be an MTG substrate (Ohtsuka et al. 2000a). In contrast Gundersen et al. (2014) showed that even very short-chain alkyl based amino acids such as glycine can serve as acceptor substrates although at reduced reactivity. Esterification of e.g. glycine and leucine increased their reactivity drastically. Herein, the reactivity decreases with decreasing carbon chain length as well as decreasing distance of the primary amine to a negatively charged substituent (e.g. carboxylate group). Further, a non-conjugated aromatic compound close to the primary amine can improve the substrate properties compared to alkyl amine. Secondary amines are no MTG substrates (Gundersen et al. 2014; Ohtsuka et al. 2000b).

Glutamin substrate peptides (Q-tags) were especially developed in the context of antibody labeling. An overview is given in section 8.

## 6 The Food Enzyme

In food industry, microbial transglutaminase (MTG) produced by fermentation is widely used to improve the physical properties like firmness, viscosity, elasticity, water-binding capacity and taste of various protein containing foods. Application areas are milk and dairy products; meat processing, sausages and hams; fish and shrimps; and pasta and bakery products (Table 3).

Table 3: Applications of MTG in food industry. Table adapted from Kieliszek and Misiewicz 2014 and de Góes-Favoni and Bueno 2014.

Source	Products	Effects	References
Meat	Restructured Meat	Improved texture	Kuraishi et al. 1997 and 2001
	PSE <sup>1</sup> meat	Greater stability	Motoki and Seguro 1998
	Emulsified, precast (burgers)	Increased hardness	Trespalcacios and Pla 2007
			de Ávila et al. 2010 Katayama et al. 2006
Fish	Surimi	Improved texture	Téllez-Luis et al. 2002
	Fish filets	Increased hardness	Vácha et al. 2006
		Greater gelling ability	Benjakul et al. 2003
Milk	Yoghurts	Improved quality and texture	Lauber et al. 2000
	Cheese	Greater viscousness	Şanlı et al. 2011
	Cream	Higher gel stability	Bönisch et al. 2007
	Deserts	Lower syneresis	Jaros et al. 2007
	Milk drinks	Better consistency	Cozzolino et al. 2003
	Dressings		
Casein	Cross-linked protein	Allergenicity reduction	Lauber et al. 2000
			Ozer et al. 2007
Wheat	Baked foods	Improved texture	Gerrard et al. 2001
		Higher dough volume and stability	Gujral and Rosell 2004
		Increased strength of gluten	
		Improved bread crump structure	
Soy	Soy protein	Higher emulsifying power	Tang 2007
	Tofu	Greater emulsion stability	Tang et al. 2006
	Mousse	Higher water holding capacity	Yasir et al. 2007
	Fermented beverages	Greater thermal stability of gels	
		Improved texture	
		Increased foam stability	
		Reduction of syneresis	
Gelatin	Sweet foods	Low calorie foods with good texture and elasticity	Giosafatto et al. 2012

1: PSE = pale, soft and exudative

*Blog by Noriki Nio and Keiichi Yokoyama (2017)*

In tertiary and quaternary structures of protein molecules, bonds and interactions such as hydrogen bond, disulfide bond, electrostatic, and van der Waal's hydrophobic interactions, greatly contribute to their formation and maintenance. Especially in food proteins, not only these, but also other bonds, particularly those found in connective tissues, are very important for the texture and mouth feeling.

The functional properties of food proteins may be changed by the use of specific enzymes under mild conditions. Whitaker suggested that it would be interesting to modify food proteins with transglutaminase, which can form cross-linkings through  $\epsilon$ -( $\gamma$ -glutamyl)lysine bonds in various proteins (Feeney and Whitaker 1977). Based on the hypothesis that cross-links contribute considerably to the physicochemical and functional properties of proteins, we examined the feasibility of the enzyme transglutaminase (TGase) in terms of protein polymerization, gelation, film formation, and peptide incorporation into proteins (Yokoyama et al. 2004).

### **1. Feasibility study on protein modification using guinea pig liver TGase**

In the early 1980s, parallel to our study, the possibility of modification of functional properties in milk caseins and soybean globulins was demonstrated using transglutaminase derived from guinea pig liver or bovine plasma. In these studies, cross-linking proteins of different origins as well as incorporation of amino acids or peptides to improve nutritional deficiencies were shown.

Concurrently, we were investigating the feasibility of food protein modification for industrial application using the guinea pig liver enzyme (mammalian tissue transglutaminase, TG2), and whey proteins, beef, pork, chicken, and fish actomyosins as substrates for the transglutaminase reaction. We discovered that these proteins formed gels by the reaction with TGase in highly concentrated solutions, e.g. soybean 11S globulin 10%, 7S globulin 10%, and  $\alpha$ S1-casein 5% (Figure 19). This discovery has become a first step in the development of commercial TGase for food industrial uses. Based on these results, transglutaminase was considered potentially useful in creating proteins with new, unique functional properties. However, its limited availability and the unfamiliarity of guinea pig liver as source hindered its commercialization. In all cases, mass production of transglutaminase had been eagerly desired.

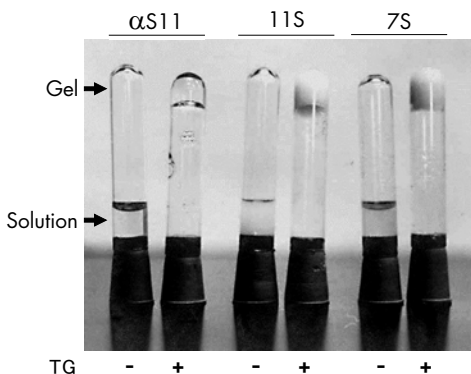


Figure 19: Gelation of protein solutions by Transglutaminase.

The proteins studied were: αS1-casein (5%, w/v); 11S globulin (10%, w/v); 7S globulin (10%, w/v). - TG, transglutaminase was not added to the protein solution; +TG, transglutaminase was added to the solution (0.03 units).

## 2. Discovery and characteristics of microbial transglutaminase (MTGase)

Although it was expected that there is a huge market for TGase applications, it was considered difficult to produce TGase in the industrial scale since it was only known to exist in mammalian organs or blood. In order to achieve commercialization of TGase, its constant supply with affordable costs was the absolute prerequisite. Therefore, screening from several thousands of microorganisms was carried out at Ajinomoto Co., Inc. in collaboration with Amano Pharmaceutical Co., Ltd. (currently Amano Enzyme Inc.).

After the intensive joint investigation, microbial TGase (MTGase) was discovered in *Streptococcus mobaraense* and its variants. These newly discovered strains had a strong activity as determined by the hydroxamate assay. The enzyme is secreted into the fermentation broth thus enabling fairly easy recovery of MTG at 42% yield. This microbial transglutaminase has its optimum activity at neutral pH but still possesses more than 30% of residual activity at either pH 4.0 or pH 9.0; MTGase is stable between pH 5.0 and 9.0. The optimum temperature for the enzymatic reaction is between 45 and 55°C and the stability was completely held at 40°C. Even a 10 minutes treatment at 50°C still retained 74% activity which revealed a relatively high temperature tolerance.

However, when the reaction mixture is heated to 70°C for just a few minutes, the enzyme is completely inactivated. These enzymatic properties are desired when applying MTGase in food processing which often includes a heating step. Most importantly the activity does not depend on calcium ions in sharp contrast to mammalian transglutaminases facilitating the use in food technology.

### 3. Structure of MTGase

Basic physicochemical properties of the enzyme such as molecular weight, amino acid sequence, predicted secondary structure and enzymatic properties have been reported in the scientific literature (Ando et al. 1989; Washizu et al. 1994). The molecular weight (MW) of MTGase was shown to be about 38 kDa in both SDS-polyacrylamide gel electrophoresis and gel-permeation chromatography. The isoelectric point (pI) was approximately 8.9. In contrast, the MW and pI of most mammalian TGases are approximately 80 kDa and 4.5, respectively. Protein sequencing by the automated Edman method and mass spectrometry revealed that the primary structure of MTGase is comprised of 331 amino acid residues. The molecular weight calculated from the amino acid sequence is 37,842, matching to the experimentally obtained value of 38 kDa. MTGase is, therefore, considered to be monomeric lacking post-translational modification (it is not a glycoprotein or lipoprotein), although there are two potential glycosylation sites (Thr-Xxx-Asn) in the primary structure. The overall sequence data indicated that MTGase has a single cysteine residue (Cys64), which is essential for the catalytic activity. The crystal structure of MTGase has been determined at 2.4 Å resolution. It forms a single, compact domain with over all dimensions 65x59x41 Å, and folds into a plate-like shape with a deep cleft at the edge of the molecule (Figure 20).

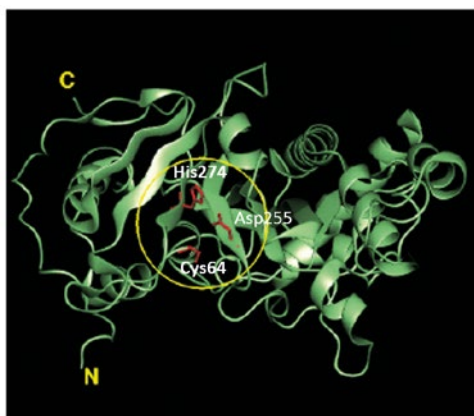


Figure 20: Structure of MTGase. Region in the yellow circle represents the active site. Red wire model shows the catalytic triad of MTGase (Cys64-Asp255-His274).



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#### 4. Bioavailability of cross-linked proteins

Because of the many promising applications of MTG-catalyzed modification of food proteins, attention should be focused on the nutritional value of resultant cross-linked proteins.

The availability of naturally occurring  $\epsilon$ -( $\gamma$ -glutamyl)lysine (abbreviated as GL) bond has been measured in raw food materials and processed food. Raw material such as meat, fish or shell fish and most processed food were found to contain certain amount of GL bond. The level of GL bond formation was relatively higher in processed foods than in raw food materials. The GL bond is also formed naturally in eggs of various fish, such as salmon, lump fish, herring, sardine, and Alaska pollack. It is still under investigation whether these naturally occurring GL bonds are formed by endogenous TG or not. However, people have consumed various foods containing GL bonds for centuries.

The digestibility of GL bond has been investigated using animal models. Within the digestion system, two enzymes namely  $\gamma$ -glutamylamine cyclotransferase and  $\gamma$ -glutamyl-transferase which cleave the GL bond are known. The lysine generated by these enzymes was shown nutritionally available to the body by animal feeding studies. In addition, the safety of the GL iso-peptide is proven also by the long-established, habitual intake of cooked foods by mankind.

The idea of a potential generation of Celiac Disease (CD)-specific deamidated gliadin epitopes resulting from MTGase-treatment of gluten-containing food was raised in 2005. It is based on the hypothesis that MTGase may mimic human tissue transglutaminase (TG2) due to the similar mode-of-action. However, it is suggested that the use of MTGase in bread and pasta preparations has no effect on the immunogenicity of gluten, but may even be used to detoxify gluten by covalent incorporation of primary amines. Recent results indicate that MTG-treatment of wheat bread and pasta prepared with typical MTGase concentrations used in standard bakery and pasta processes does not lead to immune detectable amounts of CD-immunotoxic deamidated gliadins (Ruh et al. 2014; Heil et al. 2017). Therefore, MTGase is considered as safe and is a viable option for food processing.

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## 5. Application of MTGase in food processing

As already mentioned, many food proteins are substrates for MTGase and are readily cross-linked and gelled upon incubation. The characteristics of such gelation procedure and gels formed are as follows:

- proteins that are not gelled by heating can be gelled by MTGase;
- gels that normally melt at elevated temperature no longer melt after MTGase-gelation (for example gelatin);
- protein in oil-in-water emulsions, even in the presence of sugars and/or sodium chloride, can be gelled;
- gel firmness increase after heating;
- the gels can no longer be solubilized by detergents or denaturants.

These characteristics are easily applicable for film, thread, and other forms of proteins indicating that the enzyme is very useful in protein texturization.

Prior to the practical industrial applications of MTGase, the formulations with various bulking agents were made as such that they could be handled with minimal loss of activity, especially to prevent oxidative inactivation of the thiol in the active center (Cys64). As a result, some types of MTGase-blended products were established and now commercialized for a number of practical usages for food products such as meat, fish, dairy, soybean, wheat and so on. MTGase preparations are optimized for the respective application depending on reaction conditions (concentration, pH, temperature etc.). Therefore, MTGase showed the possibility to create new protein materials in various areas as well as food applications (Figure 21).

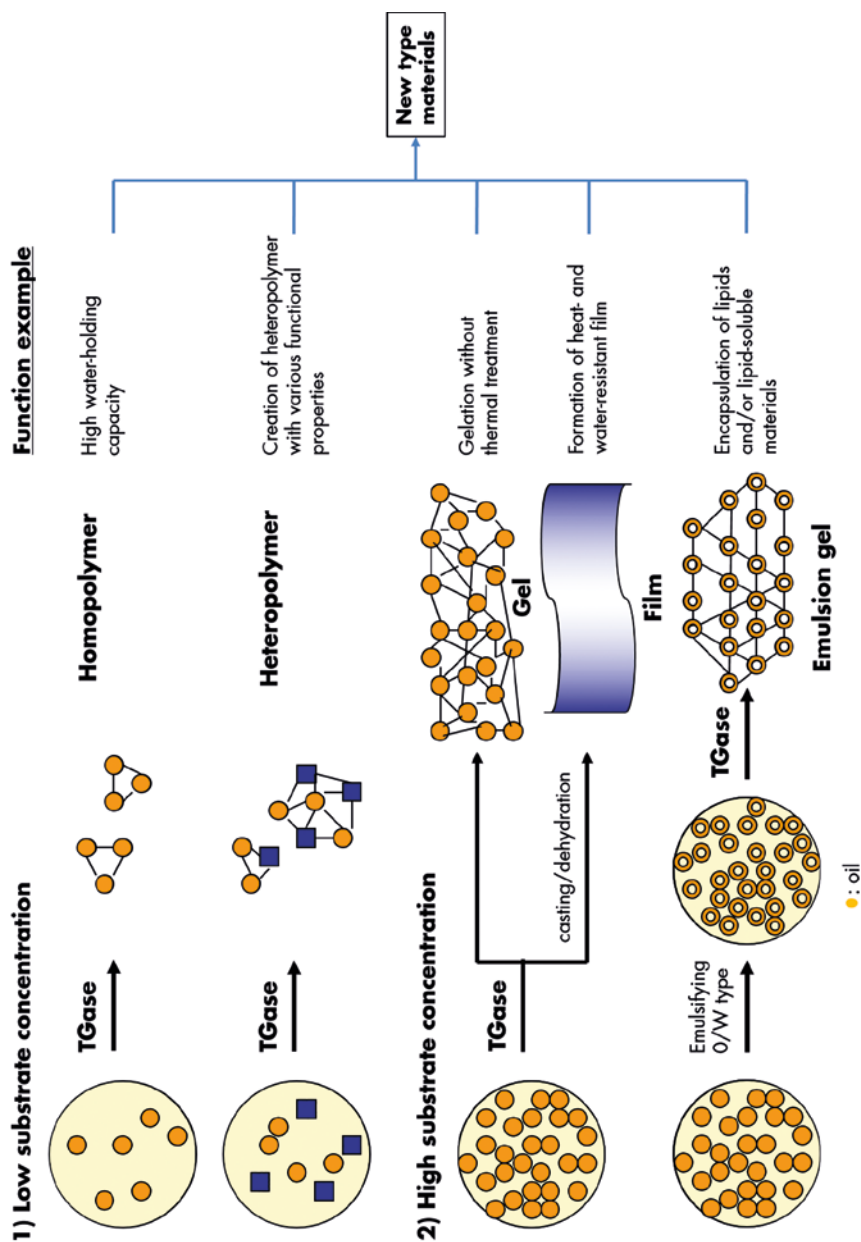


Figure 21: Utilization of cross-linking reaction by transglutaminase.

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## 6. Perspectives and conclusions

MTGase can catalytically form  $\epsilon$ -( $\gamma$ -glutamyl)lysine bonds in many proteins and such cross-links markedly alter protein function. This enzyme can be used in the development of new foods and processing methodologies. MTGase and its blended product "Actival®" have been recognized as useful for food processing and extensive safety assessment (acute and subacute toxicity, mutagenicity, etc.) has been carried out. To date, no defective effects have been reported. MTGase entered into the food market in the 1990ies. In Japan, the use of MTGase is increasing as an innovative ingredient for food processing because of its unique characteristics. GRAS notice from FDA was received in the U.S. In many other countries, MTGase is also permitted from authorities and used in food market. We are expanding the application of MTGase and hope that this enzyme will make a valuable contribution to the global food industry. MTGase is exceptional useful for generating protein based products with new and unique functional properties. There is no doubt that MTGase technology will be an essential tool for protein modification in both, food processing and non-food applications (like fine chemicals or medicinal products) in the future.

### *The authors:*



Dr. Noriki Nio retired from Ajinomoto Co. Inc., JAPAN, in 2014 and since then works as a senior advisor for Ajinomoto.

Dr. Nio studied food chemistry at Hokkaido University and joined Ajinomoto in 1980.

Since 1980, he has been engaged in utilization of transglutaminase in the food industry.



Dr. Keiichi Yokoyama works as a Bioscience & Fine Chemistry Group Manager in R&D Planning Dept. in AJINOMOTO CO., INC.

He studied microbiology and biochemistry at Tokyo University and joined Ajinomoto in 1993.

He has been engaged in several projects of developing new biochemical materials and microbial transglutaminase research for more than 10 years in Ajinomoto.

## 7 The Technical Enzyme

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Beyond the applications of MTG in food production (see section 6) technical applications are described in many fields where proteins are involved, e.g. for textile and leather processing. An overview is given by Tesfaw and Assefa (2014). However, to our knowledge so far no industrial processes using MTG in these fields are established (Tesfaw and Assefa 2014).



## 8 Site Specific Conjugation: ADCs and More

Microbial transglutaminase is a versatile tool for conjugation of proteins or peptides with small molecules, polymers, surfaces, DNA or other proteins (Strop 2014). It yields site specifically labeled, homogenous products. Many applications have been described in scientific literature, including pegylation, labeling with fluorescent dyes and conjugation of enzymes (Kamiya et al. 2009; Pasternack et al. 1998; Sato 2002; Maullu et al. 2009; da Silva Freitas et al. 2013). However, today the manufacturing of ADCs is the main application field for MTG.

### *Antibody drug conjugate formation by MTG*

An emerging field in oncology and beyond are antibody drug conjugates (ADCs). In ADC development, the aspects of antibody, payload and linker technology need to be taken into consideration (Figure 22). Each part of the ADC needs to meet high demands in order to provide high efficacy already by low drug doses, which in combination reduces the potential for adverse reactions.

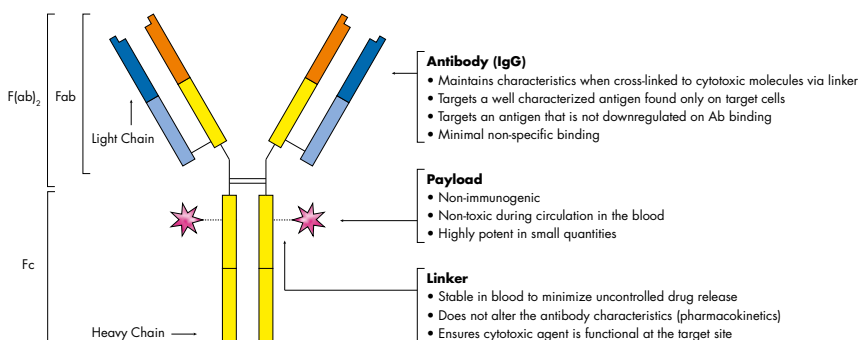


Figure 22: Schematic structure of IgG antibody drug conjugates. Figure adapted from Zolot et al. 2013 and Mulisch 2014.

Resulting from the antibody's high selectivity, the minimal effective dose can be delivered to a target (e.g. cancer cell) leading to low unspecific binding though providing the maximal tolerated dose (Schumacher et al. 2016).

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The mode of action of ADCs is based on their degradation in cell's lysosomes and subsequent payload release. To achieve that, the antibody first needs to bind the antigen at the tumor cells surface. Receptor mediated endocytosis in early endosomes imports the ADC into the cell. If the ADC's Fc-domain binds to the FcRn-receptor of the endosome, it is re-exported out of the cell. Thus, antibodies to be used as ADC should exhibit low or no binding to FcRn.

Late endosomes finally fuse with lysosomes leading to proteolytic degradation of the ADC. Depending on the selected linker technology, the payload is released either by low pH or by proteolysis. The cytotoxic payload, usually DNA-binding or microtubule polymerization inhibiting substances, leaves the lysosome and can access DNA or microtubules, leading to immediate cell death or apoptosis initiation (Peters and Brown 2015).

Drawback of this ADC mode of action can be the so called "Bystander Effect", where the released drug diffuses through the cell membrane and affects neighboring healthy cells (Bouchard et al. 2014).

ADCs can be generated using chemical, physical or enzymatic conjugation. For all methods mild reaction conditions are required, in order to maintain the antibody's native structure and functionality. Homogenous conjugates guarantee batch independent drug efficacy, required for pre-clinical and clinical development and assessment of adverse effects (Kline et al. 2015). MTG can label native antibodies, especially IgGs and can therefore be used for the generation of ADCs.

IgGs heavy chains from various species exhibit a conserved sequence of Q295[F/Y]N (Figure 23). Q295 is recognized by MTG as substrate. However, the Asp297 in proximity is glycosylated leading to sterical hindrance of MTG binding and catalysis (Figure 24).

Deglycosylation of Asp297 by the enzyme PNGase F, rendering Gln295 accessible for microbial transglutaminase, resulting in 2 labels pers antibody (1 per heavy chain, Jeger et al. 2010; Dennler et al. 2014).

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		295
		↓
Mouse IgG1	SKDDPEVQFSWFVDDDEVHTAQTPREE	QFN
Rabbit IgG	SQDDPEVQFTWYINNEQVRTARPLRE	QFN
Rat IgG	SHEDPQVKFNWYVDGVVHNAKTKPRE	QYN
Human IgG4	SQEDPEVQFNWYVDGVEVHNAKTKPRE	QFN
Human IgG3	SHEDPEVQFKWYVDGVEVHNAKTKPRE	QYN
Human IgG2	SAEDPEVQFNWYVDGVEVHNAKTKPRE	QFN
Human IgG1	SHEDPEVKFNWYVDGVEVHNAKTKPRE	QYN

Figure 23: Sequence alignment of IgG Fc-region of various species. Red: Conserved sequence Q[F/Y]N. Sequences taken from RCSB Protein Data Base. From top to bottom: 3HKF/2VUO/1FRT/5HVV/6D58/4L-4J/5D4Q. Sequence-alignment using Clustal Ω.

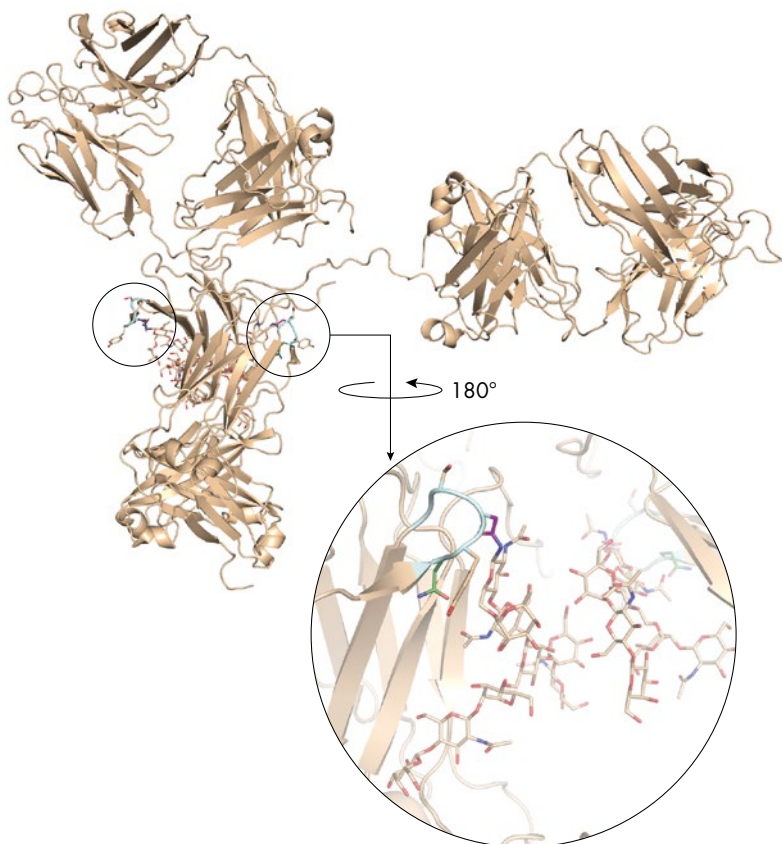


Figure 24: Crystal structure of human IgG. Enlarged: Gln295 and glycosylated Asp297 (black circle). Green: Glutamin 295; Purple: Asparagine 297; Blue: backbone of C/E-Loop QYNST. Crsytal structure from Sapphire et al., 2001 (PDB-ID: 1HZH). Programm: Pymol. (Bitsch 2016).



MTG mediated antibody conjugation is depicted in Figure 25. An in-depth description of MTG technology is given by Schibli and Spycher in the next section.

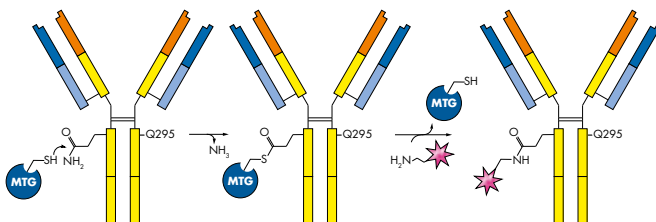


Figure 25: MTG reaction pathway. Antibody heavy chains are conjugated with a drug (linked to a primary amine) by MTG on position Q295, resulting in an ADC with two site specifically conjugated drug molecules. Here, the conjugation of only one Q295 is shown.

An alternative to deglycosylation was discovered by Spycher et al. Using lysine-containing peptides as primary amine substrates which contain an additional positively charged amino acid results in efficient labeling without deglycosylation (Spycher et al. 2019). The technology is now available at the swiss company Araris.

The Rinat-Pfizer group engineered the transglutaminase recognition sequence (Q-tag) LLQA to several positions of the heavy and light chain of IgG and successfully conjugated fluorophores and auristatin derivatives resulting in drug to antibody ratio (DAR) 1.2 - 2 (Strop et al. 2013). However, the MTG mediated conjugation lead to unspecific reactivity at Q295, which could be avoided by Q295N mutation of IgG's heavy chain (Farias et al. 2014).

The Darmstadt, Germany, based academic groups of Harald Kolmar and Hans-Lothar Fuchsbauer commonly designed Q-tag sequences based on microbial transglutaminase's natural substrates DAIP and SPI<sub>p</sub> (Siegmond et al. 2015; Ebenig 2019), showing improved reaction kinetics. Here, to avoid intermolecular cross-linking of IgG the terminal K447 had to be removed.

A further Q-tag sequence named CoviIsoLink™ was developed by the French Company Covalab (El Alaoui and Thomas 2016). They showed that sequence environment, conformation of the antibody, and type of spacer can influence the conjugation. Conjugation was improved when the Q-tag was fused to the heavy chain's C-terminus in comparison to the light chain's C-terminus (Martin et al. 2018).

An alternative to conjugation at glutamine residues was developed by Morphotek/Eisei (Spidel and Albone 2019; Spidel et al. 2017) using antibody's lysine-residues and glutamin containing peptides as substrates. In analogy to the Q-labeling approach, either native or engineered lysines can be addressed for conjugation.

*Blog by Prof. Roger Schibli and Philipp Spycher, PhD (2015)*

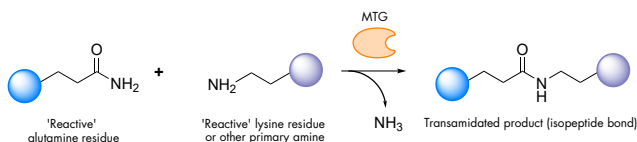
Many techniques have recently been developed to attach functional molecules to proteins either non-specifically or site-specifically. Particularly in the field of antibody-drug conjugates (ADCs) for therapeutic applications site-specific attachment of toxic compounds is a hot topic.

Using site-specific conjugation the ratio protein to attached molecule can be precisely controlled, resulting in conjugates with well-defined stoichiometry. This, for example, avoids conjugation to critical protein interaction sites and thus sustains the binding affinity of the protein. For ADCs, this results in increased tumor uptake compared to heterogeneously modified ADCs (Lhospice et al. 2015). As a site-specific technology, enzymatic conjugation has shown great interest since those conjugation reactions are typically fast and can be done under physiological conditions. Among the available enzymes, the microbial transglutaminase (MTG) from the species *Streptomyces mobaraensis* has recently been recognized as an interesting alternative to conventional chemical conjugation of chemical entities to proteins including antibodies. The MTG catalyzes under physiological conditions a transamidation reaction between a 'reactive' glutamine of a protein and a 'reactive' lysine residue whereas the latter can also be a simple, low molecular weight primary amine such as a 5-aminopentyl group (Strop 2014). For an endogenous glutamine on a protein to be recognized as an MTG-substrate two criteria seem important:

- 1) hydrophobic amino acids around the glutamine seem to foster conjugation (Sugimura et al. 2008);
- 2) the glutamine must be situated on a loop with local chain flexibility enhancing reactivity toward MTG (Spolaore et al. 2012).

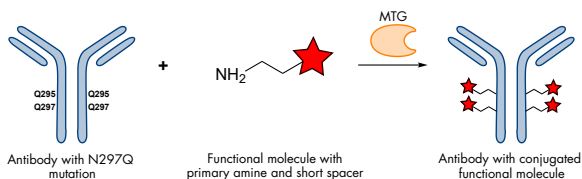
From our experience as well as from others it seems, however, that only few glutamines typically fulfill these requirements (Kamiya et al. 2003) e.g. in antibodies, thus, making the MTG an attractive tool for site-specific and stoichiometric protein modifications.

### MTG mechanism<sup>10</sup>



The Schibli group identified a single reactive glutamine on the heavy chain of different IgGs, which is recognized by MTG: Q295. Located on a loop which upon enzymatic deglycosylation of the asparagine 297 (N297) becomes flexible can easily and very efficiently be conjugated with various substrates (Jeger et al. 2010). This property allows for direct modification of the antibody in a straightforward manner. By using this approach, exactly two chemical entities can be conjugated to the antibody backbone. We furthermore discovered that by ablating the asparagine 297 through genetic mutation into a glutamine (N297Q) two more substrates could be attached generating an antibody with four covalently and homogeneously attached substrates. Incorporation of four highly toxic drugs is, for example, particularly attractive for the generation of antibody-drug-conjugates where the drug load plays a decisive role in effective tumor killing.

### Antibody conjugation using Q295 and mutation N297Q<sup>10</sup>



During our studies we have also observed that the genetically introduced myc-tag, consisting of only 10 amino acids with one being a glutamine, was efficiently recognized by the MTG. Upon sequence analysis we could determine that the reaction was indeed catalyzed by the glutamine within the myc-tag (Dennler et al. 2015). Since the myc-tag is often used as a protein identification tool after recombinant protein expression, MTG-mediated bioconjugation can thus be used in a straightforward manner to generate homogenous conjugates with one functional moiety attached at the terminal myc-tag like biotin-cadaverine, a fluorescent dye or a radiochelator. Such a biotinylation can for example be used to immobilize proteins on a solid surface, therefore providing a controlled orientation. In case of immobilized e.g. antibody fragments a controlled orientation will likely enhance interactions with their antigens compared to a random orientation where some of the interactions sites will not be accessible due to an unfavorable orientation. This can also be applied to the uncontrolled conjugation of molecules to proteins since these could as well be attached to important protein-interaction sites leading to decreased binding and lower affinity towards their designated binding partners.

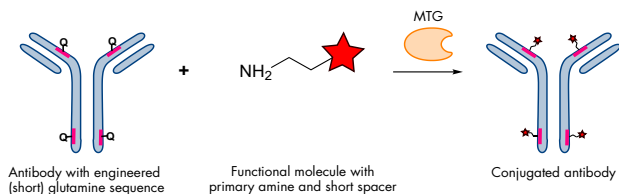
In contrast terminal attachment will likely sustain high affinity-binding as such deleterious effects will be avoided. Additionally, attaching several moieties in a non-controlled manner often changes the solubility behavior of the conjugated protein particularly when the attached moieties are bulky and hydrophobic. Altered solubility could lead to unfavorable protein-conjugate aggregation and eventually, precipitation with loss of valuable material. With MTG a controlled amount of the hydrophobic molecule is attached generating conjugates which are well solubilized yet.

Genetic tags like the myc-tag can not only be placed at protein termini but also directly within the protein's backbone.

This, for example, has been achieved for antibodies by Pfizer, where the antibody's amino acid sequence was genetically engineered such that at different positions, probes could be incorporated using MTG and a short four amino acid motif (Strop et al. 2013). This approach virtually allows conjugating any probe at any position as long as it is accessible enough and the protein structure will not be compromised upon sequence introduction. This approach thus requires careful structure analysis to evaluate and determine the least interfering locations.

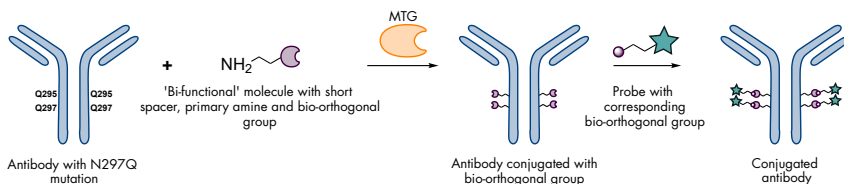
MTG can also be used for chemo-enzymatic functionalization of proteins in case the enzyme is not tolerant to a substrate resulting in a low conjugation yield. Thereby a small 'bi-functional' spacer is first conjugated to the protein consisting of a primary amine (recognized by MTG) and a reactive group suitable for subsequent bio-orthogonal coupling of functional molecules. In a second step, and after purification, the functional molecule can easily be conjugated to the already modified protein at low molecular excess. Using this approach, we could conjugate large toxins to antibodies efficiently and at low molecular excess achieving a well-defined stoichiometry (Dennler et al. 2014).

### Engineered backbone conjugation<sup>10</sup>



<sup>10</sup> Figures with courtesy of Philipp Spycher, 2015

## Chemo-enzymatic modification<sup>10</sup>



Taken together, MTG-mediated bioconjugation is a very attractive tool for the efficient generation of homogeneously modified proteins with virtually every substrate as long as it contains a primary amine. However, since MTG is somewhat promiscuous towards the glutamine and lysine residues with no defined substrate preference, it would be desirable to develop MTGs with altered substrate specificities such that different molecules could be conjugated which will be the task for future research.

### *The authors:*



**Prof. Roger Schibli** is an Associate Professor in the Department of Chemistry and Applied Biosciences (ETH Zurich) since 2010, and Head of the Center for Radiopharmaceutical Science (CRS), a joint endeavor between the ETH Zurich, the Paul Scherrer Institute and the University Hospital Zurich. Prof. Schibli's research interests cover targeted tumor diagnosis and therapy using radio-labeled compounds. Apart from the chemical and enzymatic modification and radioactive labeling of molecules, his group emphasizes on the biological and pharmacological characterization and optimization of the radioactive compounds including non-invasive PET and SPET imaging.



**Philipp Spycher, PhD** obtained his Master's Degree and Ph.D. from ETH Zurich (Switzerland) at the interface of Material Science and Protein Engineering. During his post-doctoral work at the Paul Scherrer Institute (PSI, Switzerland), he introduced the novel approach using transglutaminases for antibody conjugation that led to the discovery of the Araris Linker Technology. Philipp won the PSI Founder Fellowship as well as several other prizes and grants to commercialize the technology. He is now leading Araris as CEO and managed to assemble a world-class team of co-founders and co-workers.

*Blog by Philipp Spycher, PhD (2020)*

Antibody-drug conjugates (ADCs) consist of three components: the antibody (mediates tumor-targeting) the payload (drug) and the linking-moiety (linker) that connects the drug to the antibody. ADCs are thought to selectively deliver drugs to tumors or other targets while the healthy tissue is spared, enabling thereby a so-called targeted chemotherapy.

Despite this simple concept, the field has faced major challenges with regard to efficacy and safety. In recent years, tremendous progress has been made leading to 7 approved ADCs and numerous ADCs to be approved in near future. Still, current ADCs and ADC technologies face three major challenges:

- 1) challenging and costly ADC assembly,
- 2) linker instability leading to unacceptable toxicities and reduced efficacy,
- 3) limited solubility of the linker resulting in non-optimal drug properties. In fact, many of the clinical ADC failures could be attributed to one or more of the above-mentioned weaknesses.

### **The Araris difference**

At Araris Biotech AG we are developing a novel linker technology to address these challenges. Our technology is based on novel lysine-peptide linkers and microbial transglutaminase (MTG)-mediated conjugation to the antibody. Using our lysine-peptide linkers we can conjugate the payload directly to the Q295 residue of fully glycosylated (native) antibodies without any prior antibody engineering step. The hydrophilic nature of our linkers furthermore helps to accommodate the (often) hydrophobic payloads and keep them well-solubilized resulting in ADCs with favorable physicochemical properties.

The conjugation can be done in 1-step whereby the linker-payload is directly conjugated to the antibody (Figure 26A, upper panel). Alternatively, the payload can be conjugated in two steps with the conjugation chemistry being flexible, like azide(N3)-click-chemistry or thiol-maleimide (Figure 26A, lower panel). Due the peptidic nature of the linker, the drug-load can be easily increased from two (DAR2) to four attachment sites (DAR4) using a branched linker with a single-attachment site on the native antibody (Figure 26B). Different conjugation chemistries can also be provided on the same linker, like azide and thiol, enabling thus the conjugation of two different payloads (DAR 2+2) (Figure 26B).

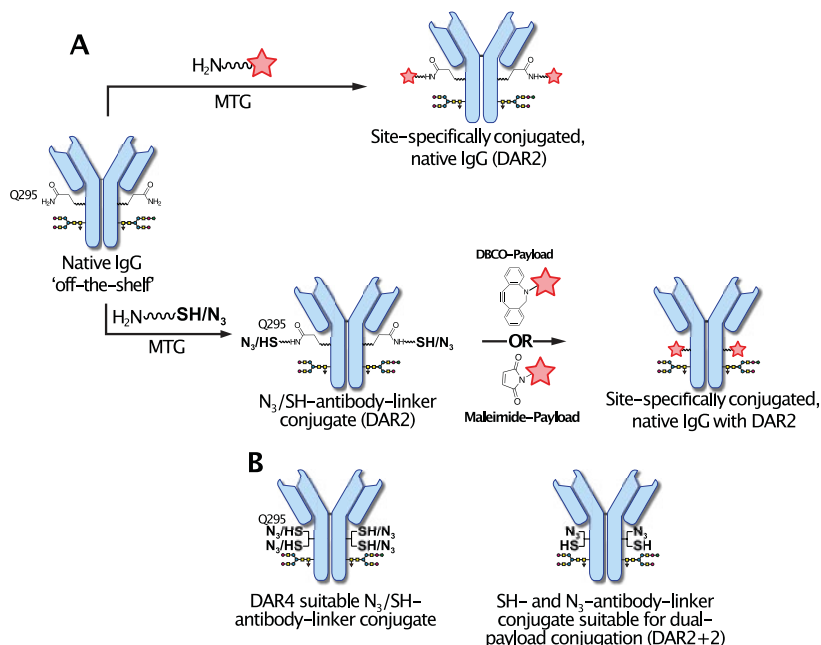


Figure 26: Using a peptide-linker containing a lysine residue enables site-specific MTG-mediated conjugation to Q295 of native antibodies without prior antibody engineering. The payload can be conjugated in a 1- or 2-step process, by either conjugating the linker-payload to the antibody or first installing a reactive handle on the antibody followed by payload conjugation (A). The peptidic nature thereby allows to install various chemistries such as azide (N<sub>3</sub>) for click-chemistry or thiols for maleimide conjugation. By branching the linker two conjugation sites can be incorporated enabling thus a drug-load of 4 (DAR4) using e.g. N<sub>3</sub> or Thiol (B). Both types of chemistries can also be installed on the same linker for dual-payload conjugation (DAR2+2).

In summary, our results indicate that the ADCs generated with our linker technology have favorable biophysical properties, are stable and result in very efficient anti-tumor responses in efficacy studies.

Thus, ADCs generation with the Araris Linker Technology, offers the advantage that any native and unmodified antibody can be used 'off-the-shelf'. ADCs can be generated in as little as 48 hours and are highly stable and efficacious. Due to the hydrophilic nature of the linker and its chemical versatility it is well suited to accommodate all payloads. At Araris we thus believe that we can offer ADCs to patients with an improved therapeutic window.

*The author:*

**Philipp Sypher, PhD**, Profile see previous section, page 35.

## 9 How to Measure and Detect MTG

Kits for measuring MTG-activity as well as for detection of MTG-protein are available at Zedira.

### *Standard transglutaminase activity assay (Hydroxamate-assay)*

Today's microbial transglutaminase standard assay principle was introduced already in 1950 by Heinrich Waelsch and co-workers (Grossowicz et al. 1950) and applied for guinea pig liver transglutaminase in 1966 by Folk and Cole. The assay uses the small molecule Z-Gln-Gly-OH as the glutamine donor substrate and hydroxylamine as amine substrate. In the presence of active microbial transglutaminase, hydroxylamine is incorporated into Z-Gln-Gly-OH forming Z-glutamyl-hydroxamate-glycine which, under acidic conditions, develops a red colored complex with iron (III) detectable at 525 nm (Figure 27) (Folk and Cole 1966).

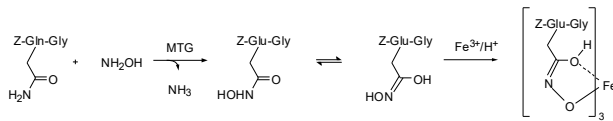


Figure 27: MTG mediated incorporation of hydroxylamine into Z-Gln-Gly-OH forming Z-glutamyl-hydroxamate-glycine. The hydroxamate side-chain forms a red colored complex with iron (III) ions.

The assay is available at Zedira as ZediXclusive Microbial Transglutaminase Assay Kit (Z009). The kit shows a linear increase of  $\Delta E_{525}$  from 0.1 to 2.8 U/mL microbial transglutaminase (Figure 28). Measuring higher activity values requires sample dilution. The lower detection limit (LDL) of this assay is 3  $\mu\text{g/mL}$  MTG assuming the lowest measurable  $\Delta E_{525}$  with 0.1 U/mL and a specific activity of MTG with 30 U/mg.

The assay is characterized by its robustness and reliability. The chromogenic endpoint assay is easy to use with short measurement time. A validation protocol is available.

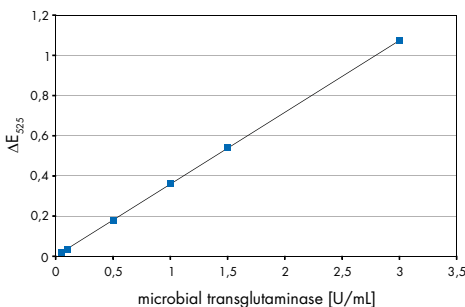


Figure 28: Assay linearity of ZediXclusive Microbial Transglutaminase Assay Kit (Z009).



## Ammonium-NicotinamidADPH-GLDH-Transglutaminase Assay (ANiTA)

An alternative method for determination of MTG activity is the MTG-ANiTA-Kit (M001), which uses  $\beta$ -Casein as high molecular weight substrate. MTG catalyzes the formation of isopeptide bonds between protein-bound glutamine and lysine releasing one ammonium molecule per isopeptide bond formed. In the indicator reaction of the MTG-ANiTA-Kit the amount of released ammonium is monitored in a glutamate dehydrogenase (GLDH) catalyzed NADPH-dependent reaction. The consumption of NADPH is measured spectrophotometrically by the decrease of absorbance at 340 nm (Figure 29).

As this assay is based on a protein substrate it is especially suitable for the characterization of MTG-variants.

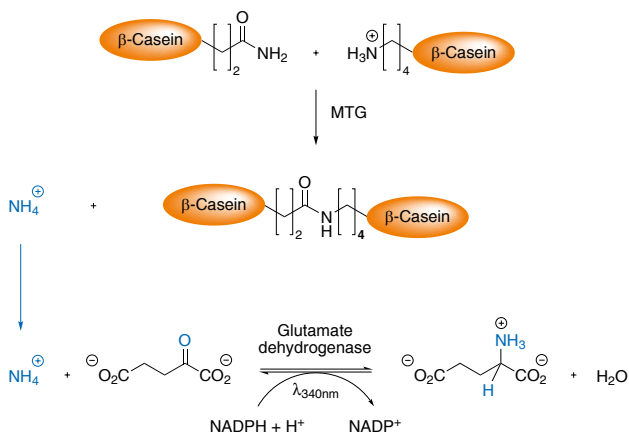


Figure 29: Principle of MTG-ANiTA-Kit (M001).

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## Microbial transglutaminase sandwich ELISA

ZediXclusive Microbial transglutaminase (MTG) ELISA (E021) is a sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantitative determination of microbial transglutaminase from *Streptomyces mobaraensis* (MTG). It may also be used for the detection of MTG in food samples like meat, sausage, yoghurt, quark, cheese and others.

The detection of MTG with the kit requires sample preparation which includes homogenization and centrifugation.

Quantification of MTG in samples is possible using a calibration curve based on the MTG-standards included in the kit.

Lower MTG-Detection Limit: 0.6 ng/mL (LDL)

Limit of MTG-Quantitation: 3.0 ng/mL (LoQ)

3 ppb (1 mL = 1 g)

Please note that Lower MTG-Detection Limit of the kit is reduced for denatured MTG by factor 20.

### Principle of the test

The microtiter plate wells (solid phase) are coated with antibodies specific to microbial transglutaminase.

- 1<sup>st</sup> reaction: Microbial transglutaminase present in the sample binds to the immobilized antibodies, forming the antibody-antigen-complex.
- 2<sup>nd</sup> reaction: A microbial transglutaminase specific antibody labeled with biotin binds to the antibody-antigen-complex forming an antibody-antigen-[antibody-biotin] sandwich.
- 3<sup>rd</sup> reaction: Streptavidin conjugated to horseradish peroxidase (HRP) binds to the biotin-label for the antibody-antigen-[antibody-biotin] sandwich.
- 4<sup>th</sup> reaction: The HRP-labeled complex converts a substrate (TMB) into a blue product which upon addition of the stop solution turns yellow. Samples containing microbial transglutaminase develop the blue color (which upon addition of the stop solution turns yellow), whereas samples without microbial transglutaminase remain colorless.

## Measuring microbial transglutaminase in food

Microbial transglutaminase is used as processing aid in food industry in order to improve the quality, e.g. of sausage, pasta, bread and yoghurt (see section 6).

Zedira provides the tools to determine MTG activity (Z009) and MTG protein (E021) in complex samples like MTG raw materials, formulations and food samples in easy-to-use kit formats.

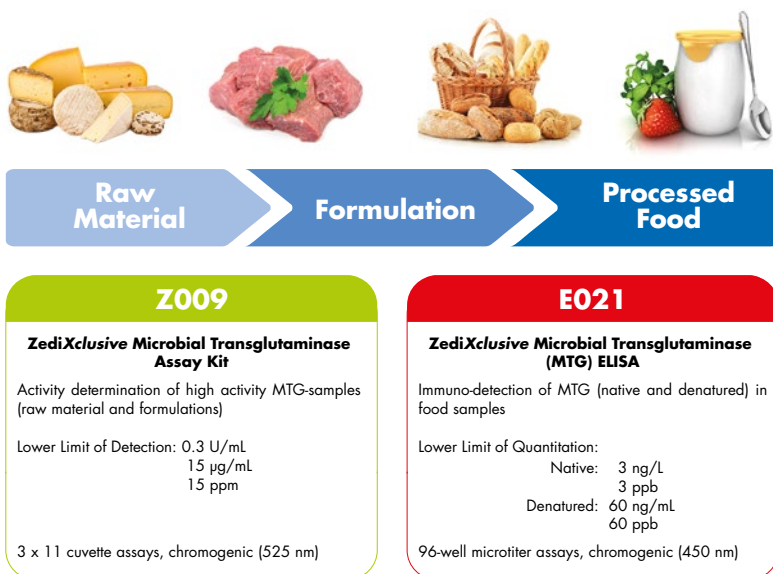



Figure 30: Kits for MTG-activity determination and MTG-protein detection in food industry processing steps. Z009 is suitable for analyzing MTG raw materials and formulations. E021 can be used for detection of MTG in processed food samples.

## 10 MTG-Products and Tools

Next to the above-mentioned assays and ELISAs, Zedira offers the whole toolbox for working with MTG: Enzymes, antibodies, inhibitors and substrates. Please have a look at the following pages and explore our broad MTG portfolio. And always keep in mind contacting us whenever you need a tool you do not find here.

### Microbial (Pro)Transglutaminase

Synonym: Bacterial transglutaminase, MTG, BTG

Art. No.	Name	Unit	Price
<b>T001</b>	<b>Microbial</b> (bacterial) transglutaminase	<b>25 U</b>	<b>270 €</b>
	(MTG, recombinantly produced in <i>E. coli</i> ,	<b>250 U</b>	<b>2140 €</b>
	gene derived from <i>Streptomyces mobaraensis</i> )	<b>10 x 25 U</b>	<b>2140 €</b>
		<b>500 U</b>	<b>3750 €</b>
		<b>20 x 25 U</b>	<b>3750 €</b>
			
<b>T016</b>	<b>Microbial</b> (bacterial) <b>Pro</b> -transglutaminase (Pro-MTG, recombinantly produced in <i>E. coli</i> , gene derived from <i>Streptomyces mobaraensis</i> )	<b>250 µg</b>	<b>270 €</b>
<b>T178</b>	<b>Microbial</b> transglutaminase with <b>C-terminal His<sub>6</sub>-Tag</b>	<b>25 U</b>	<b>370 €</b>
	(MTG-His <sub>6</sub> , recombinantly produced in <i>E. coli</i> ,	<b>10 x 25 U</b>	<b>2950 €</b>
	gene derived from <i>Streptomyces mobaraensis</i> )	<b>20 x 25 U</b>	<b>5150 €</b>

References: Kaempffe et al., J. Pharm. Sci. 2021, S0022-3549(21)00400-7;  
 Früh et al., ACS Nano 2021, 15, 12161–70;  
 Stricker et al., J. Pediatr. Gastroenterol. Nutr. 2019, 68:e43-e50 ;  
 Spycher et al., ChemBioChem 2017, 18:1923-7;  
 Steffen et al., J. Biol. Chem. 2017, 292:15622-35;  
 Dennler et al., Chembiochem. 2015, 16:861-7;  
 Dennler et al., Bioconjugate Chem. 2014, 25, 569-78;

Zedira has been awarded ISO 9001:2015 certification,  
one of the most rigorous and well-regarded standards in the world.



Art. No.	Name	Unit	Price
<b>T153</b>	<b>Andracon™</b> , recombinant microbial transglutaminase	<b>25 U</b>	<b>370 €</b>
	(production grade)	<b>10 x 25 U</b>	<b>2950 €</b>
		<b>20 x 25 U</b>	<b>5150 €</b>

### Special features of **Andracon™**

- Recombinantly produced in *E. coli*
- Ultra-pure and highly active
- Batch to batch consistent quality
- Scientific and technical support
- Available from 25 U vials up to bulk amounts
- License free
- Produced according to an SOP in an ISO9001:2015 certified environment
- Characterized for host cell protein, host cell DNA, and endotoxin-levels
- Customer audits for qualification according to guideline ICH Q7 possible

Kaufmann et al., Food Addit. Contam. Part. A 2012, 29:1364-73;  
Jeger et al., Angew. Chem. Int. Ed. Engl. 2010, 49:9995-7;  
Gianfrani et al., Gastroenterology 2007, 133:780-9;  
Pfleiderer et al., Microbiol. Res. 2005, 160:265-71;  
Ando et al., Agric. Biol. Chem. 1989, 53:2613-17;  
Pasternack et al., Eur. J. Biochem. 1998, 257:570-6

## Antibodies to Microbial Transglutaminase

Art. No.	Name	Unit	Price
<b>A020</b>	<b>Polyclonal</b> antibody to microbial protransglutaminase (pro-MTG)	<b>500 µg</b>	<b>365 €</b>
	Immunogen: Bacterial protransglutaminase (T016), raised in rabbit		
	Format: Purified IgG		
<b>A024</b>	<b>Monoclonal</b> antibody to <b>microbial</b> transglutaminase	<b>100 µg</b>	<b>365 €</b>
	Immunogen: Bacterial transglutaminase (T001), raised in mouse		
	Format: Ascitic fluid, lyophilized		
<b>A051</b>	<b>Biotinylated</b> polyclonal antibody to microbial transglutaminase (MTG)	<b>50 µg</b>	<b>365 €</b>
	Format: A019 purified via affinity chromatography on protein A, followed by biotin-labeling		
<b>A143</b>	<b>Monoclonal</b> antibody to microbial transglutaminase (clone XM67, IgG1)	<b>200 µg</b>	<b>365 €</b>
	Immunogen: Microbial protransglutaminase (T016), raised in rabbit		
	Format: Purified IgG, recommended as <b>capture antibody</b>		
<b>A144</b>	<b>Monoclonal</b> antibody to microbial transglutaminase (clone XM68, IgG2a)	<b>200 µg</b>	<b>365 €</b>
	Immunogen: Microbial protransglutaminase (T016), raised in rabbit		
	Format: Purified IgG, recommended as <b>detection antibody</b>		
<b>A145</b>	<b>Polyclonal</b> antibody to microbial transglutaminase	<b>200 µg</b>	<b>365 €</b>
	Immunogen: Microbial protransglutaminase (T016), raised in rabbit		
	Format: Purified IgG		
	Note: A145 replaces polyclonal antibody A019		

### Amine-donor substrates (Q-Labeling)

Suitable substrates for labeling of your target molecule with accessible glutamine residues (Q-Labeling).

#### Biotin Label

Art. No.	Name	Unit	Price
<b>B002</b>	<b>N-(Biotinyl)cadaverine</b>	<b>25 mg</b>	<b>160 €</b>
		<b>100 mg</b>	<b>420 €</b>

#### Click-Chemistry Labels

Art. No.	Name	Unit	Price
<b>A152</b>	<b>3-Azidopropan-1-amine</b>	<b>100 mg</b>	<b>160 €</b>

Application: Amine donor substrate for transglutaminase, suitable for click chemistry

<b>A153</b>	<b>4-Azidobutan-1-amine</b>	<b>100 mg</b>	<b>160 €</b>
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Application: Amine donor substrate for transglutaminase, suitable for click chemistry

#### PEG Labels

Art. No.	Name	Unit	Price
<b>P010</b>	<b>(PEG1,088)amine</b> , monodisperse	<b>10 mg</b>	<b>160 €</b>

Application: Polyethylene glycol amine substrate of transglutaminase

<b>P011</b>	<b>(PEG5,000)amine</b> , polydisperse	<b>10 mg</b>	<b>160 €</b>
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Application: Polyethylene glycol amine substrate of transglutaminase

#### Fluorescent Labels

Art. No.	Name	Unit	Price
<b>A106</b>	<b>(ATTO-488™)cadaverine</b>	<b>1 mg</b>	<b>250 €</b>

<b>A112</b>	<b>(ATTO-532™)cadaverine</b>	<b>1 mg</b>	<b>250 €</b>
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<b>A113</b>	<b>(ATTO-550™)cadaverine</b>	<b>1 mg</b>	<b>250 €</b>
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<b>A114</b>	<b>(ATTO-647N™)cadaverine</b>	<b>1 mg</b>	<b>300 €</b>
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## Fluorescent Labels continued

Art. No.	Name	Unit	Price
<b>A115</b>	<b>(ATTO-700™)cadaverine</b>	<b>1 mg</b>	<b>300 €</b>
<b>D006</b>	<b>N-(Dansyl)cadaverine</b>	<b>100 mg</b>	<b>160 €</b>
<b>K004</b>	<b>“KxD”, Boc-Lys-en-DNS</b>	<b>10 mg</b>	<b>240 €</b>
<b>R001</b>	<b>N-(Tetramethylrhodaminy)cadaverine,</b> <b>N-(TAMRA)cadaverine</b>	<b>10 mg</b>	<b>160 €</b>

Reference: Dennler et al., Chembiochem. 2015, 16:861-7;  
Lee et. al., J. Biotechnol. 2013, 168:324-30;  
Kasprzak et al., Biochemistry 1988, 27:4512-22

## Glutamine-donor peptides (K-Labeling)

Suitable substrates for labeling of your target molecule with accessible lysine residues (K-Labeling).

### Biotin Label

Art. No.	Name	Unit	Price
<b>C091</b>	<b>Z-Gln-Gly-CAD-Biotin</b>	<b>25 mg</b>	<b>315 €</b>

Reference: Spidel et al., Bioconjugate Chem. 2017, 28:2471-84;  
Zindel et al., PLoS One 2016, 11:e0149145;  
Pasternack et al., Anal. Biochem. 1997, 249:54-60

## Click-Chemistry Labels

Art. No.	Name	Unit	Price
<b>C079</b>	<b>Z-Gln-Gly-PEG(3)-N<sub>3</sub></b>	<b>25 mg</b>	<b>475 €</b>

Application: Glutamine donor substrate for transglutaminase, suitable for click chemistry

<b>C080</b>	<b>Z-Gln-Gly-PEG(11)-N<sub>3</sub></b>	<b>25 mg</b>	<b>475 €</b>
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Application: Glutamine donor substrate for transglutaminase, suitable for click chemistry



## PEG Labels

Art. No.	Name	Unit	Price
<b>C092</b>	<b>Z-Gln-Gly-(PEG1,088)</b> , monodisperse	<b>25 mg</b>	<b>475 €</b>
Application: Polyethylene glycol glutamine substrate of transglutaminase			
<b>C093</b>	<b>Z-Gln-Gly-(PEG5,000)</b> , polydisperse	<b>25 mg</b>	<b>475 €</b>
Application: Polyethylene glycol glutamine substrate of transglutaminase			

## Fluorescent Labels

Art. No.	Name	Unit	Price
<b>C002</b>	<b>Z-Gln-Gly-CAD-DNS</b>	<b>25 mg</b>	<b>160 €</b>
		<b>100 mg</b>	<b>350 €</b>
<b>C074</b>	<b>Z-Gln-Gly-CAD-(ATTO-488™)</b>	<b>1 mg</b>	<b>375 €</b>
<b>C075</b>	<b>Z-Gln-Gly-CAD-(ATTO-532™)</b>	<b>1 mg</b>	<b>375 €</b>
<b>C076</b>	<b>Z-Gln-Gly-CAD-(ATTO-550™)</b>	<b>1 mg</b>	<b>375 €</b>
<b>C077</b>	<b>Z-Gln-Gly-CAD-(ATTO-647N™)</b>	<b>1 mg</b>	<b>475 €</b>
<b>C078</b>	<b>Z-Gln-Gly-CAD-(ATTO-700™)</b>	<b>1 mg</b>	<b>475 €</b>
<b>C090</b>	<b>Z-Gln-Gly-CAD-TAMRA</b>	<b>25 mg</b>	<b>475 €</b>
Reference: Spycher et al., ChemBioChem 2017, 18:1923-7			

## Inhibitor

Art. No.	Name	Unit	Price
<b>C102</b>	<b>MTG-Blocker</b>	<b>10 mg</b>	<b>210 €</b>
Application: Inhibition of microbial transglutaminase, IC <sub>50</sub> ~ 125 µM		<b>25 mg</b>	<b>385 €</b>
Reference: Spycher et al., ChemBioChem 2017, 18:1923-7; Rickert et al., Protein Sci. 2016, 25:442-5			

## 11 Application Notes

### Protein Labeling

Labeling of proteins using microbial transglutaminase is a smart alternative to chemical protein-labeling procedures. Because of transglutaminase substrate requirements generally a lower number of labels is achieved – compared to chemical labeling – resulting in improved product properties:

- Defined degree of labeling
- Defined label position(s)
- Homogenously labeled protein
- Higher solubility in water
- Minimized amounts of unlabeled protein
- No or reduced impact on bioactivities of labeled proteins



Figure 31: Principle of MTG-catalyzed protein labeling.

Transglutaminase labeling requires substrate sequences on the target protein surface, which are in general not abundant on proteins.

Therefore, in a first step the target protein must be analyzed for its MTG substrate properties. This can easily be achieved with Zedira's Substrate Finder Kit (L001).

The target protein is accessible to MTG labeling, if it contains either glutamine-substrate sequence(s) or lysine substrate sequence(s) or both.

If the target protein contains neither glutamine- nor lysine-substrate sequences, MTG-substrate tags may be introduced by recombinant techniques.

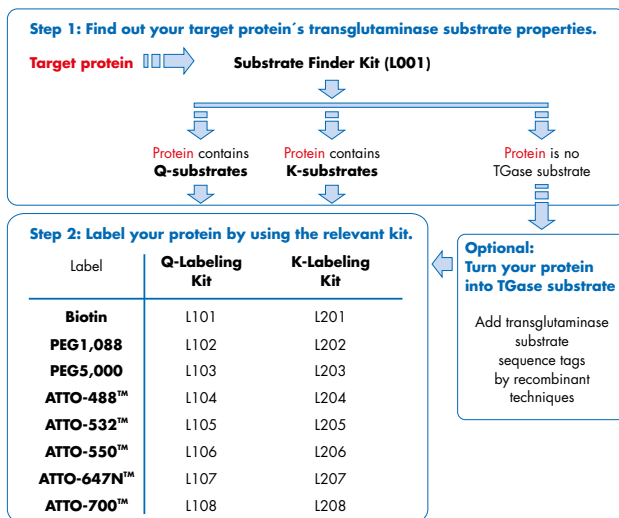
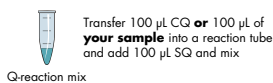


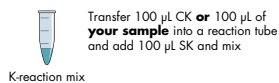
Figure 32: Substrate Finder Kit based choice of labeling kit.

### Reaction mix preparation

#### Labeling of glutamine residues (Q-Labeling)



#### Labeling of lysine residues (K-Labeling)



### Assay procedure

#### Identical steps for Q- and K-Labeling

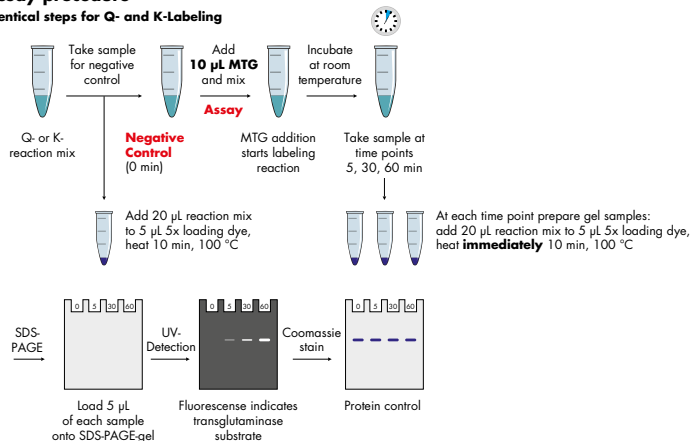


Figure 33: Schematic application of Substrate Finder Kit.

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## PEGylation of proteins

Protein PEGylation mediated by microbial transglutaminase (MTG) was performed using the positive control proteins of Substrate Finder Kit (L001).

### Q-Labeling

Control protein CQ (Q-substrate Control Protein) contains an amino acid tag rendering the protein to a glutamine-donor protein. Labeling of CQ with amine-donor (PEG5,000)cadaverine is shown in Figure 34.

The molecular weight shift of CQ (28 kDa) indicates PEG-derivate incorporation with increasing reaction time. Over time, the band of CQ decreases, whereas a band at 40 kDa appears. After 16 h of incubation, no QC band is visible indicating complete PEGylation with the respective derivate.

It can be noted that PEGylation of CQ requires longer incubation times than the dansylation utilized in the Substrate Finder Kit (L001).

Note: It is not possible to label CK with amine-donors, since CK does not contain any accessible glutamine residues.

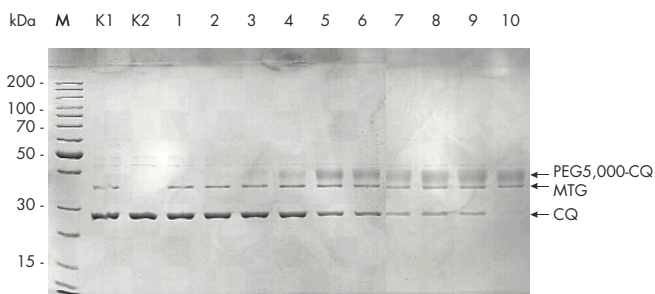


Figure 34: MTG mediated incorporation of PEG derivate (PEG5,000)cadaverine into CQ using L103. Reaction mixture (RM) contained CQ, PEG derivate and MTG and was incubated at 37°C on a shaker. At several time points samples were taken, mixed with 5x loading dye and heated immediately. Samples were analyzed by SDS-PAGE followed by Coomassie staining. M: Molecular weight marker; K1: RM without PEG derivatives; K2: RM without MTG; lanes 1-10: Samples taken after 5 min, 15 min, 30 min, 60 min, 120 min, 180 min, 240 min, 300 min, 360 min and 16 h reaction time. Arrows: MTG is indicated as well as CQ and PEGylated CQ.

## K-Labeling

Control protein CK (K-substrate Control Protein) contains an amino acid tag rendering the protein to an amine-donor protein. Labeling of CK with glutamine-donor Z-Gln-Gly-(PEG5,000) is shown in Figure 35.

The tag used in CK contains several lysin residues. Thus, a higher number of PEG molecules labeled to this protein can be expected.

The molecular weight shift of CK (29 kDa) indicates PEG derivate incorporation with increasing reaction time. Over time, the band of CK decreases, whereas several bands at ~60 kDa and higher molecular weights appear.

Already after 15 min of incubation, no CK band is visible indicating complete PEGylation with the PEG5,000 derivate. This increased cross-linking reaction can be attributed to the structure and accessibility of the tag used for CK.

Note: It is not possible to label CQ with glutamine-donors, since CQ does not contain any lysin residues.

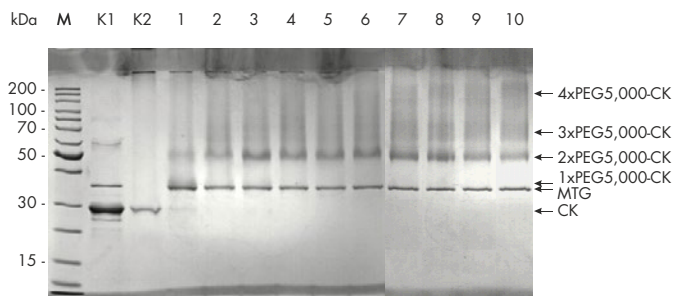


Figure 35: MTG mediated incorporation of PEG derivate Z-Gln-Gly-(PEG5,000) into CK using L203. Reaction mixture (RM) contained CK, PEG derivate and MTG and was incubated at 37°C on a shaker. At several time points samples were taken (20 µl), mixed with 5x loading dye (5 µl) and heated immediately. Samples were analyzed by SDS-PAGE followed by Coomassie staining. M: Molecular weight marker; K1: RM without PEG derivatives; K2: RM without MTG; lanes 1-10: Samples taken after 5 min, 15 min, 30 min, 60 min, 120 min, 180 min, 240 min, 300 min, 360 min and 16 h reaction time. Arrows: MTG is indicated as well as CK and PEGylated CK.

## Labeling of Antibodies

ADC manufacturing using MTG is well described in the literature, summarized in section 8 of this MTG-Handbook. Below, basic examples for antibody labeling by MTG are shown.

### Q-Labeling of human IgG with fluorescent substrate Boc-Lys-en-DNS ("KxD", K004).

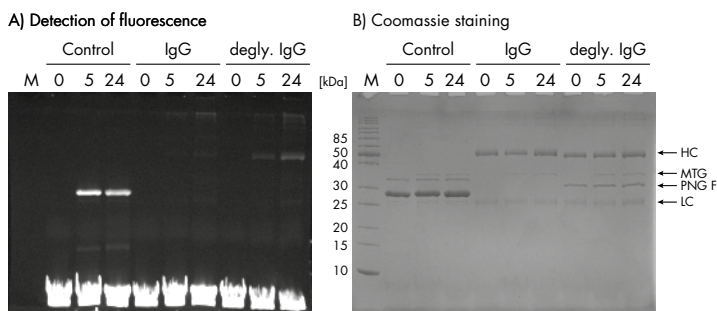


Figure 36: Q-Labeling of glycosylated and deglycosylated human IgG, SDS-PAGE analysis. 100  $\mu$ g human IgG was labeled in the presence of 300  $\mu$ M Boc-Lys-en-DNS at 37°C by 0.5 U/ml MTG. Samples were taken after 0, 5 and 24 h after MTG addition. Control = Control protein fused with Q-Tag (MTG substrate sequence); IgG = human serum IgG; degly. IgG = deglycosylated human serum IgG; PNG F = PNGase F; HC = antibody heavy chain; LC = antibody light chain; M = PageRuler™ Unstained Protein Ladder. A) Detection of fluorescence by UV excitation; B) Coomassie stained PAGE-Gel (12.5%).

As shown in Figure 36, labeling of glycosylated IgG heavy chain was weak after 24 h reaction time while deglycosylated IgG was labeled well after 5 h incubation. In both cases the 24 h sample showed higher fluorescence signal intensity revealing the need for longer reaction time. Labeling of antibody light chain was not observed, as expected.

### Variation of MTG volume activity

Literature describes labeling of antibodies using up to 6 U/mL MTG (Dennler et al. 2014). In the following, dansylation of deglycosylated human serum IgG in the presence of 300  $\mu$ M Boc-Lys-en-DNS was performed by varying MTG volume activity (0.5 – 10 U/mL).

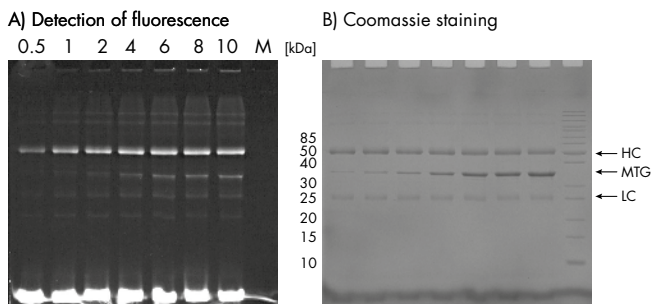


Figure 37: Variation of MTG activity in IgG-labeling, SDS-PAGE analysis. 40  $\mu$ g deglycosylated human serum IgG was labeled in the presence of 300  $\mu$ M Boc-lys-en-DNS by 0.5-10 U/ml MTG, incubated at 37°C for 24 h. HC = antibody heavy chain; LC = antibody light chain; M = PageRuler™ Unstained Protein Ladder. A) Detection of fluorescence by UV excitation; B) Coomassie stained PAGE-Gel (12.5%).

As expected, the fluorescence signal intensity increased with increasing MTG concentration. Furthermore, auto-labeling of MTG was observed starting with 6 U/mL as shown in Figure 37 (protein band at ~ 38 kDa).

Further experiments showed similar labeling efficiency from 37°C to 48°C. The optimal pH for dansylation of human serum IgG by microbial transglutaminase is pH 6-7.

### Chemo-enzymatic labeling of proteins and antibodies

MTG-conjugation combined with “click-chemistry” enables easy and fast coupling of reaction partners in order to achieve stereospecific and uniform conjugates.

Activation of antibodies with a “click-chemistry” partner can be catalyzed by microbial transglutaminase using a click-chemistry substrate carrying a primary amine function.

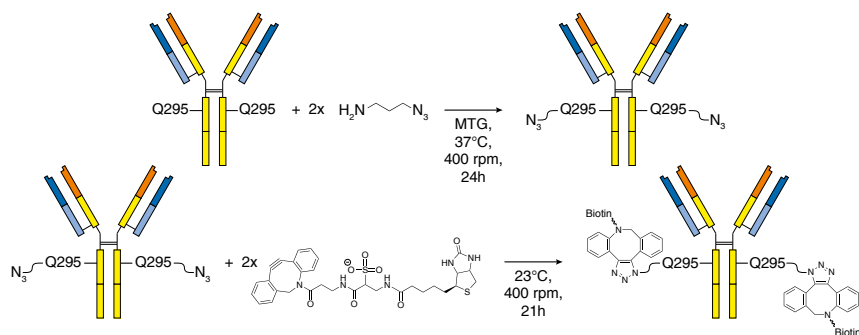


Figure 38: Schematic representation of chemo-enzymatic conjugation of antibodies. First step, enzymatic conjugation of 3-Azidopropan-1-amine (Zedira, A152) to the deglycosylated antibody catalyzed by MTG. Second step, click-reaction of azide-labeled antibody with Sulfo-DBCO-Biotin.

In Figure 39 an SDS-PAGE analysis of the MTG catalyzed conjugation of deglycosylated IgG with 3-Azidopropan-1-amine (top) or 4-Azidobutan-1-amine (bottom) is shown; followed by clicking with Sulfo-DBCO-Biotin for 21 h at 23°C.

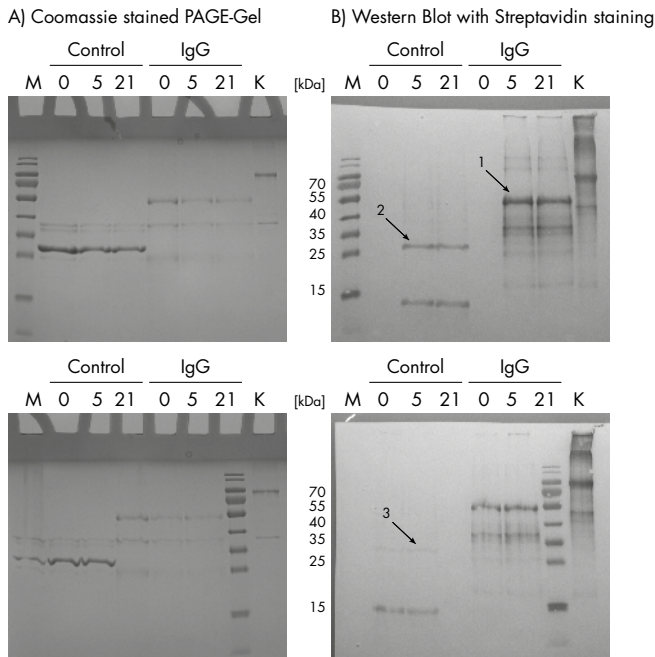


Figure 39: Chemo-enzymatic biotinylation of deglycosylated antibody. A) Coomassie stained PAGE gels; B) Western Blot followed by Streptavidin detection. Top: 3-Azidopropan-1-amine; Bottom: 4-Azidobutan-1-amine. Sampling time: 0, 5 or 21 h at 37°C; Control = q-tagged control protein; M = PageRuler™ prestained protein ladder; K = biotinylated control protein; Arrow 1 = biotinylated heavy chain of IgG; Arrow 2/3 = biotinylated control.



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## *Protocol for MTG-catalyzed antibody conjugation*

Note: the antibody deglycosylation protocol as well as the conjugation protocol should be optimized for each antibody and antibody-conjugate. The protocol below (courtesy of Philipp Spycher, PSI, revised) provides a starting point.

### **Antibody deglycosylation**

1. Dissolve antibody in PBS or 0.1 M Tris pH 7.6 (other buffers might work as well) at a concentration of 1 mg/mL (other antibody concentrations can be used too for deglycosylation).
2. Add 6 U PNGase F per mg of antibody.
3. Incubate overnight (16 h) at 37°C.
4. Check successful deglycosylation with LC-ESI-MS (reduce antibody with DTT for MS-analysis).

Note: Each (iso-)type of antibody behaves differently towards deglycosylation (e.g. different kind of glycosylation due to different expression systems used etc.), therefore the above mentioned parameters might need to be adjusted individually (e.g. increased PNGase F concentration, extended incubation time, etc.). For some antibodies, deglycosylation may result in increased aggregation.

### **Antibody Conjugation**

Deglycosylated antibody reaction mixtures obtained according to the protocol above can directly be used for MTG conjugation, usually no buffer exchange is necessary. PNGase will be removed later. For initial experiments use antibody concentrations of 1 mg/mL, in PBS or Tris (as above).

For batch production: use higher antibody concentration.

---

## Conjugation Protocol

1. Reconstitute MTG (T001, Zedira) by the addition of the volume of pure water indicated as aliquotation volume in the respective CoA.
2. Prepare an MTG-stock solution (50 U/mL) by addition of pure water.
3. Add 6 U/mL to the 1 mg/mL antibody solution.
4. Add substrate (e.g., biotinylcadaverine, B002, Zedira) to the mixture, mix well and centrifuge down.  
Final substrate concentration: 80 molar excess to antibody (0.533  $\mu$ M substrate for 1 mg/mL antibody).  
Note: do not exceed 5% DMSO.
5. Incubate overnight (16 h) at 37°C.
6. Stop conjugation reaction by either:
  - a. Removing excess substrate (and MTG) with ultra-centrifugal filters (>50 kDa MWCO).
  - b. Addition of MTG-blocker from Zedira (C102) to the reaction mixture (1 mM, >30 min, 37°C).
7. Analyze conjugation efficiency with LC-ESI-MS (reduce antibody with DTT for MS-analysis)  
Mass = MW (heavy chain of antibody) + MW of substrate – 17 Da (due to release of  $\text{NH}_3$ ).  
Note: For fluorescent substrates analysis may be performed by fluorescence spectrometry.
8. Removal of MTG for batch production may be performed by size exclusion chromatography (e.g. Superdex 200 10/300 GL).

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Structure of microbial transglutaminase from *S. mobaraensis* (PDB code 1IU4).

## Print

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*Targeting*  
**Transglutaminase**  
*for research and medicine!*

Microbial transglutaminase (MTG) is an enzyme originally produced by *Streptomyces mobaraensis*. MTG catalyzes cross-linking of proteins in the absence of calcium. Current application field of the enzyme is processing of meat, sausage, dairy, bakery, and pasta products to improve food properties.

However, MTG cannot only cross-link proteins, it can also incorporate primary amines to proteins in a covalent, irreversible manner. This feature allows linking of a whole bundle of labels to proteins, including biotin, fluorescent dyes, click chemistry substrates and cytotoxins.

MTG's unique properties can be utilized to generate site specific and homogenous antibody drug conjugates (ADCs). Today, ADCs manufactured with MTG reached clinical trials.

Zedira's MTG-Handbook shall provide an overview on this fascinating enzyme. This 2<sup>nd</sup> edition of Zedira's MTG-Handbook includes additional information on MTG's characteristics useful for the everyday work in the lab: pH- and temperature profile, stability and more. In addition, a starting protocol for antibody conjugation is included.



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