3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

MGMT ASSAY KIT
Product Number **MD0100**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

MGMT (O^6-Methylguanine-DNA methyltransferase) is a ubiquitous DNA repair protein that removes O^6-alkyl-guanine lesions, primarily O^6-methyl-guanine, from damaged DNA. It is a major contributor to cellular protection from the mutagenic, carcinogenic, and cytotoxic effects of DNA alkylation. The mechanism of MGMT action is based on the transfer of the alkyl group from the DNA to a unique acceptor cysteine residue in the protein, forming a stable thioether linkage. The cysteine sulfhydryl moiety is not regenerated; therefore, MGMT is frequently classified as a suicide protein.[1-3] Since the reaction irreversibly inactivates the enzyme, the repair capacity for O^6-methylguanine is dependent on the number of MGMT molecules in the cell. There is a correlation between the occurrence of cancer in various tissues and the lack of the MGMT enzyme.[4,5] On the other hand, high levels of MGMT, found occasionally in tumors, result in reduced tumor events and resistance of tumors to alkylating agents.[2] Therefore, MGMT is a target for both over-expression studies aimed to protect hematopoietic stem cells from cancer,[6] and inhibition drug discovery studies aimed to be used in conjunction with oncologic alkylating agents.[7]

The MGMT assay kit is based on the formation of a new restriction site in a 23-mer ^{32}P-labeled double-stranded (ds) oligodeoxynucleotide. The new site is created by the removal of the methyl group from an O^6-methylguanine residue by the MGMT enzyme. Cleavage of the new restriction site and separation of its products on a denaturing PAGE gel produces a fragment of 8 bases in addition to the original 23 base oligonucleotide. Detection is performed by autoradiography.[8]

The kit can be used for:

- Screening for MGMT inhibitors.
- Detection of MGMT activity in cell lines.

Components/Reagents

The kit contains reagents sufficient for 100-200 tests.

MGMT Substrate O^6-Methylguanine strand
Product Code M 5817 — 1 vial
23-mer oligodeoxynucleotide, 200 pmol
GAA**CTLCAG**CTCCGTGCTGGCCC
L = O^6-Methyl-dG

MGMT Substrate complement strand
Product Code M 5692 — 1 vial
23-mer oligodeoxynucleotide, 260 pmol
GGGCCAGCACGGAG**CTGCAG**TTC

10X Reaction Buffer,
Product Code R 7277 — 1.5 ml
500 mM HEPES, pH 7.5, 500 mM KCl,
0.25% Triton X-100, 10 µg/ml BSA

Stop Solution
Product Code S 1568 — 1 ml
90% Formamide, 0.1% w/v Bromophenol blue,
0.1% Xylene cyanole, 20 mM EDTA

Methylguanine-DNA Methyltransferase (MGMT)
Product Code M 8065 — 10 µg
Human recombinant, expressed in *E. coli*.
200-400 µg/ml in 50% (w/v) glycerol containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, and 200 mM NaCl.
Activity: >10,000 units per mg protein
Unit definition: One unit removes 50% of the methyl groups from 0.1 pmol ds-DNA oligonucleotide substrate containing one O^6-methylguanine in 10 min. at 37 °C.

O^{6-}Benzylguanine solution
Product Code B 6809 — 50 µl
10 mM in DMSO

Spin column-10
Product Code S 2045 — 2 each

Equipment and reagents needed but not supplied

- Molecular biology grade water, Product Code W 4502, or autoclaved ultrapure (17 mΩ·cm) water.
- Microcentrifuge tubes - autoclaved.
- ATP-[γ-^{32}P] 10 mCi/ml.
- T4 polynucleotide kinase (PNK), Product Code P 4390.
- 10x T4 polynucleotide kinase (PNK) buffer: 100 mM NaCl, 50 mM Tris-HCl, 10mM $MgCl_2$, 1 mM DTT, pH 7.9, 25 °C).
- Pst I, restriction endonuclease, Product Code R 7023.
- 10x Pst I buffer, Product Code B 3657.
- Tris-Borate-EDTA buffer (TBE) Product Code T 9525.
- 20% 7 M urea PAGE gel.[9]
- Microcapillary round tips, Product Code T 1906 (optional).
- Autoradiography film.
- Automatic processor for autoradiography film or supplies for manual processing.

Additional reagents required for determination of MGMT activity in cell lysates

- PBS (Dulbecco's phosphate buffered saline, Product Code D 8537).
- Lysis buffer: 50mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 5% Glycerol, 50 mM NaCl, and 1 mM AEBSF.
- Phenol – chloroform – isoamyl alcohol (25:24:1), Product Code P 3803.
- Transfer RNA (tRNA), Product Code R 9001.
- 3 M Sodium Acetate, pH 5.2, Product Code S 7899.
- Ethanol, 100% and 90% (cold).
- Sonicator.

Precautions and Disclaimer

Sigma's MGMT assay kit (MD0100) is for laboratory use only and not for drug, household or other use. Consult the MSDS for information regarding hazards and safe handling practices.

Storage/Stability

Store the kit at –20 °C. After opening, the spin columns can be stored at room temperature.

Procedure

Assay Principle

The activity of MGMT is determined by measuring the extent of methyl group removal from a mutated 23-mer double-stranded oligodeoxynucleotide containing O^6-methylguanine within a unique Pst I restriction site. The MGMT substrate strand, containing O^6-Methylguanine, is end-labeled with ^{32}P and annealed to its complement (MGMT substrate complement strand). MGMT removes the methyl group from O^6-methylguanine, creating a Pst I recognition site. Upon incubation with Pst I restriction endonuclease, the repaired substrate is cut to 8- and 15-mer oligonucleotides. The products are separated on a denaturating 7 M urea PAGE gel and analyzed by autoradiography. The non-repaired substrate migrates as a 23-mer oligonucleotide. The repaired substrate migrates as an 8-mer oligonucleotide.

The kit can be used for assaying MGMT enzyme activity or for screening MGMT inhibitors (Procedure A) or for determining MGMT activity in cell lysates (Procedure B).

Substrate preparation

Note: The substrate components in this kit are sufficient for performing 2 labeling reactions, each sufficient for more than 100 MGMT activity assays.

Use molecular biology grade water or autoclaved ultrapure (17 mΩ·cm) water.

1. Reconstitute the MGMT Substrate O^6-Methylguanine strand (first strand oligodeoxynucleotide) with 24 µl water. Store the unused oligodeoxynucleotide solution at –20 °C.
2. Reconstitute the MGMT substrate complement strand with 30 µl water. Store the unused oligodeoxynucleotide solution at –20 °C.
3. Warm Spin column-10 to room temperature.

Labeling of first strand oligodeoxynucleotide.

1. Assemble the following reaction mix:

Compound	Volume
10x PNK buffer	3 µl
First strand oligonucleotide	10 µl (100 pmol)
ATP [γ-^{32}P] 10 mCi/ml	3 µl (30 µCi)
T4 PNK	1 µl
H_2O	13 µl
Total:	30 µl

2. Incubate for 60 min. at 37 °C.
3. Stop the reaction by incubating for 10 min. at 70 °C.
4. Remove the unreacted ATP from the sample using Spin column-10 as follows:
   - Invert the spin column several times to suspend the matrix uniformly.
   - Snap off the tab at the tip.
   - Place the lower end of the column into a 2 ml microcentrifuge tube.
   - Centrifuge the column 5 min. at 700 x *g*.

- Discard the collection tube containing the equilibration buffer.
- Place the column in a second microcentrifuge tube.
- Carefully apply the reaction sample to the center of the flat surface of the matrix bed.
- Centrifuge at 700 x *g* for 5 min. to collect the labeled oligodeoxynucleotide in the collection tube. The expected volume obtained is 35-45 µl.
- Discard the spin column.

Annealing

1. Add 13 µl of the complement strand oligdeoxynucleotide to the labeled first strand oligdeoxynucleotide (Section a).
2. Anneal strands by the following incubations: 1 min. at 95 °C, 5 min. at 37 °C, and 30 min. at room temperature.
3. Store labeled substrate at –20 °C in a storage box for β-emitters.

MGMT Activity Assay and Screening for MGMT Inhibitors

For screening MGMT inhibitors, MGMT is first incubated with an inhibitor. Following this incubation step, the residual activity of MGMT is determined. For determination of MGMT activity only, skip the preincubation step and use increasing amounts of MGMT in the assay.

Reagents preparation for 10 activity assay reactions

Use molecular biology grade water or autoclaved ultrapure (17 mΩ·cm) water.

Enzyme dilution buffer

Dilute 2 µl of 10x Reaction Buffer in 38 µl of water.

MGMT enzyme

Dilute MGMT to 25 µg/ml using Enzyme Dilution Buffer. Calculate the dilution factor from the MGMT specific activity. Prepare 25-30 µl.

O^6-benzylguanine (MGMT inhibitor) 100µM

Dilute 1µl 10 mM O^6-Benzylguanine in 100 µl of water.

Inhibitor of interest

Prepare various dilutions of the tested inhibitor. Make sure that the DMSO concentration in the sample does not exceed 4% (2% in the preincubation step).

Reaction mix

Mix 10 µl of 10x Reaction Buffer, 2 µl of ^{32}P-labeled substrate, and 48 µl water.

Pst I mix

Mix Pst I restriction enzyme with 10x Pst I buffer at a 1:1 ratio. Prepare 25 µl just before use.

Denaturing gel[9]

Prepare a 20% denaturing gel containing 7 M urea, and pre-run the gel for 30-60 min. at 100 V.

Reaction scheme

	Step 1				Step 2
	MGMT 25 µg/ml	**Tested Inhibit.**	**O^6Benzyl guanine** 100 µM	**H_2O**	**Reaction mix**
Positive control	2 µl	----	--	2 µl	6 µl
Test	2 µl	2 µl	----		6 µl
Negative control	2 µl	----	2 µl		6 µl
Blank	---	----	----	4 µl	6 µl

Preincubation

1. Add 2 µl of 25 µg/ml MGMT enzyme to the microcentrifuge tubes (see Reaction scheme).
2. Add 2 µl of tested inhibitor solution (Test), or 2 µl water (Positive control), or 2 µl O^6-Benzylguanine solution (Negative control).
3. Add 4 µl water to the Blank tube (no enzyme).
4. Mix by pipetting and cap the tubes.
5. Incubate for 30 min. at 37 °C.
6. Transfer the tubes to an ice bath.

MGMT activity reaction and Pst I restriction assay

1. Add 6 µl of Reaction mix to each tube.
2. Mix by pipetting.
3. Incubate for 10 min. at 37 °C.
4. Incubate for 5 min. at 65 °C (to inactivate the MGMT).
5. Incubate on ice for 10 min.
6. Add 2 µl of the Pst I mix to each tube.
7. Incubate for 60 min. at 37 °C.
8. Stop reactions by adding 5 µl of Stop Solution.
9. Heat for 5 min. at 95 °C.
10. Keep the samples on ice.

Denaturing SDS-PAGE

1. Load 5-7 µl of each sample on the gel using micro-capillary round tips (optional). Before loading, wash the wells with running buffer.
2. Run the gel at 100-200 V until the bromophenol blue dye (dark blue) reaches 1 cm from the front (bromophenol blue and xylene cyanole run as approximately 10- and 28-base oligonucleotides, respectively).

3. Carefully disassemble the gel and lay it on a piece of Whatman 3MM paper. Cover the gel with a piece of saran wrap.
   Note: do not let the gel dry as it may crack.
4. Expose the gel to X-ray film for 1-3 hours at –70 °C.

Determination of MGMT activity in cell lysates
The procedure detailed below is suitable for HeLa cells. For other cell lines, it is recommended to determine the amount of protein required for MGMT activity detection.

Reagents preparation for 10 activity assay reactions
Use molecular biology grade water or autoclaved ultrapure (17 mΩ·cm) water.

Lysis buffer
50 mM Tris, pH 7.5, 1mM EDTA, 1mM DTT, 5% Glycerol, 50 mM NaCl and 1 mM AEBSF. Prepare 1 ml (if the Lysis Buffer is also required for cell lysate preparation, prepare 10 ml).

Enzyme dilution buffer
Dilute 1 µl of Reaction Buffer 10x in 19 µl of water.

MGMT positive control
Dilute MGMT enzyme to make 25 µg/ml using Enzyme dilution buffer. Calculate the dilution factor according to the specific MGMT enzyme concentration. Prepare 8-10 µl.

Reaction mix
Add 2 µl of ^{32}P-labeled substrate to 100 µl Reaction buffer 10x.

Pst I mix
Mix PstIPst I restriction enzyme with 10x Pst I buffer at a 1:1 ratio. Prepare 25 µl just before use.

tRNA
Prepare a solution of 1 mg/ml

Denaturing gel[9]
Prepare a 20% denaturing gel containing 7 M urea and pre-run the gel for 30-60 min.

Cell Lysate. Use 70-350 µg protein of HeLa cell lysate per test. (For other cell lysates, test up to 1 mg protein.)

Cell lysate preparation
1. Harvest HeLa cells (approx. 5 x 10^8).
2. Collect cells by centrifugation at 1000 x *g* for 5 min. (approx. 1 ml packed cells).
3. Wash the cells 4-5 times with PBS.
4. Add 4-5 ml of Lysis Buffer to the cell pellet.
5. Freeze at –80 °C for 2 hours.
6. Thaw the cells at room temperature.
7. Sonicate 4 times for 10 seconds each at 4-10 °C, using a microtip at maximum energy.
8. Centrifuge at 14,000 x *g* for 30 min.
9. Collect the supernatant and determine protein concentration (expected concentration: 4-7 mg/ml)
10. Save the supernatant and store in aliquots at –80 °C.

Reaction scheme

	Cell Lysate	**MGMT** 25µg/ml	**Lysis Buffer**	**Reaction mix**
Test	x µl	---	88-x µl	12 µl
Blank	---	---	88 µl	12 µl
Positive Control	---	2 µl	86 µl	12 µl

x is the volume of cell lysate tested.

MGMT reaction
1. Add x µl of lysate (70-350 µg protein) to a microcentrifuge tube (Test).
2. Complete volume to 88 µl with lysis buffer.
3. Add 88 µl of lysis buffer to the Blank control tube.
4. Add 2 µl of 25 µg/ml MGMT to the Positive control tube and complete volume to 88 µl with lysis buffer.
5. Add 12µl of reaction mix to each of the tubes.
6. Mix by pipetting and cap the tubes.
7. Incubate for 60 min. at 37 °C.
8. Incubate for 5 min. at 65 °C (to stop the activity of MGMT).

Substrate extraction and precipitation
1. Add 100 µl of phenol – chloroform – isoamyl alcohol (25:24:1) reagent to each of the tubes.
2. Vortex vigorously for 10 sec.
3. Centrifuge for 3 min. at 14,000 x *g*.
4. Transfer the upper aqueous phase to a new microcentrifuge tube.
5. Add 100 µl water to the organic layer to re-extract.
6. Vortex vigorously for 10 sec.
7. Centrifuge for 3 min. at 14,000 x *g*
8. Collect the upper aqueous phase and combine with the first extraction. Discard the organic fraction.
9. Add 10 µl of 1mg/ml tRNA (10 µg) to the aqueous fractions, to serve as a carrier.
10. Add 25 µl of 3M Sodium Acetate, pH 5.2. Vortex.
11. Add 600 µl of cold absolute ethanol.
12. Incubate at –20 °C for 30 min.
13. Centrifuge for 6 min. at 13,000 x *g*.
14. Carefully aspirate the liquid.
    Note: the pellet may be invisible.
15. Add 1 ml of cold 90% ethanol to the tubes.
16. Centrifuge for 6 min. at 13,000 x *g*.
17. Carefully aspirate the liquid.

Note: the pellet may be invisible.

18. Inverse the microcentrifuge tube on a filter paper for 10-15 min. to dry the pellet.

Pst I restriction assay and denaturing SDS-PAGE

1. Add 8 µl water to the bottom of each tube.
2. Add 2 µl of the Pst I mix to each tube.
3. Incubate for 60 min. at 37 °C.
4. Stop reactions by the addition of 5 µl of Stop Solution.
5. Heat for 5 min. at 95 °C.
6. Keep the samples on ice.
7. Load 5-7 µl of each sample on the gel using micro-capillary round tips (optional). Wash the wells with running buffer before loading.
8. Run the gel at 100-200 V until the bromophenol blue dye (dark blue) migrates to 1 cm from the front (bromophenol blue and xylene cyanole run as approximately 10- and 28-base oligonucleotides, respectively).
9. Carefully disassemble the gel and lay it on a piece of Whatman 3MM paper. Cover the gel with a piece of saran wrap.
   Note: do not let the gel dry, as it may crack.
10. Expose to X-ray film for 1-3 hours at –20 °C. It is recommended to put two sheets of film on the gel in order to get at least one gel properly exposed.

References

1. Hansen, W. K., and Kelly, M. R., Review of mammalian DNA repair and translational implications. J. Pharmacol. Exp. Ther., **295**, 1-9 (2000).
2. Margison, G. P., and Santibanez-Koref, M. F., O^6-alkylguanine-DNA alkyltransferase: Role in carcinogenesis and chemotherapy. BioEssays, **24,** 255-266 (2002).
3. Sassanfar, M., et al., Relative efficiencies of the bacterial, yeast, and human DNA methyltransferases for the repair of O^6-methylguanine and O^4-methylthymine. Suggestive evidence for O^4-methylthymine repair by eukaryotic methyltransferases. J. Biol. Chem., **266**, 2767-2771 (1991).
4. Silber, J. R., et al., Lack of the DNA repair protein O^6-methylguanine-DNA methytransferase in histologically normal brain adjacent to primary human brain tumors. Proc. Natl. Acad. Sci. USA, **93,** 6941-6946 (1996).
5. Lees, N. P., et al., Longitudinal variation in O^6-alkylguanine DNA-alkyltransferase activity in the human colon rectum. Br. J. Cancer, **87**, 169-170 (2002).
6. Jansen, M., et al., Hematoprotection and enrichment of transduced cells in vivo after gene transfer of $MGMT^{P140K}$ into hematopoietic stem cells. Cancer Gene Ther., **9**, 737-46 (2002).
7. KuDOS Pharmaceuticals, www.kudospharma.co.uk/r_d/patrin.php
8. Encell, L. P., and Loeb, L. A., Redesigning the substrate specificity of human O^6-alkylguanine-DNA transferase. Mutants with enhanced repair of O^4-methylthymine. Biochemistry, **38**, 12097-12103 (1999).
9. *Current Protocols in Molecular Biology*, Ausubel, F. M., et al. (Eds.) p. 12.2.3 (John Wiley, New York, 2000).

JWM 5/29/03