

## 6xHis MsrB2 Protein aa: 42-182

(Human recombinant)

Cat. # MB201

Lot:

Upon arrival, store at 4°C (desiccated)

See datasheet for storage after reconstitution

<b>Form:</b>	Lyophilized white powder
<b>Amount of material:</b>	2 x 50 µg
<b>Validated applications:</b>	Actin Reduction at Met 44, Met 47

Online Datasheet Contains  
MB201 (V1.9) and MB201-XL(V1.9)

### Background Information

Recently, it was shown that the MICAL protein can interact with F-actin to promote oxidation at Met44 and Met47<sup>[1]</sup> (see Figure 1). Subsequent studies discovered that MICAL-mediated methionine sulfoxide (MetO) of actin is reversed by the SelR/MsrB family of methionine sulfoxide reductase (Msr) enzymes<sup>[2,3]</sup>. The Msr family are well conserved proteins that function to reduce oxidized methionine to L-methionine<sup>[4]</sup>.

Two groups independently identified SelR (MsrB) as the enzyme responsible for reduction of the Met 44 and Met47 amino acids of actin; importantly, this reduction of Met44 and Met47 was sufficient to restore normal actin polymerization<sup>[2,3]</sup>. These studies determined that the CxxS catalytic cysteine motif was essential for MsrB effects, as mutating the cysteine residue abolished MsrB's ability to restore actin polymerization<sup>[2]</sup>. These studies also determined that SelR/MsrB selectively reduced MICAL-oxidized actin, while another methionine sulfoxide reductase member, MsrA, did not<sup>[2,3]</sup>. Since SelR/MsrB specifically reduces R-isomer (MetO) and MsrA specifically reduces S-isomer MetO the groups concluded that MICAL stereo-specifically oxidized actin with MetO R-isomer. Collectively, these data describe a reversible, specific redox system that controls actin dynamics and cytoskeletal organization through regulation of a specific MetO of actin.

### Material

Human MSRB2 protein (MB201) [accession # Q9Y3D2 (MSRB2\_HUMAN)] has been produced and purified from a bacterial expression system. The recombinant protein is N-terminally 6x Histidine tagged and is comprised of amino acids 42-182<sup>[5]</sup>. MB201 has an approximate molecular weight of 17 kDa. MB201 is supplied as a white lyophilized powder.

### Storage and Reconstitution

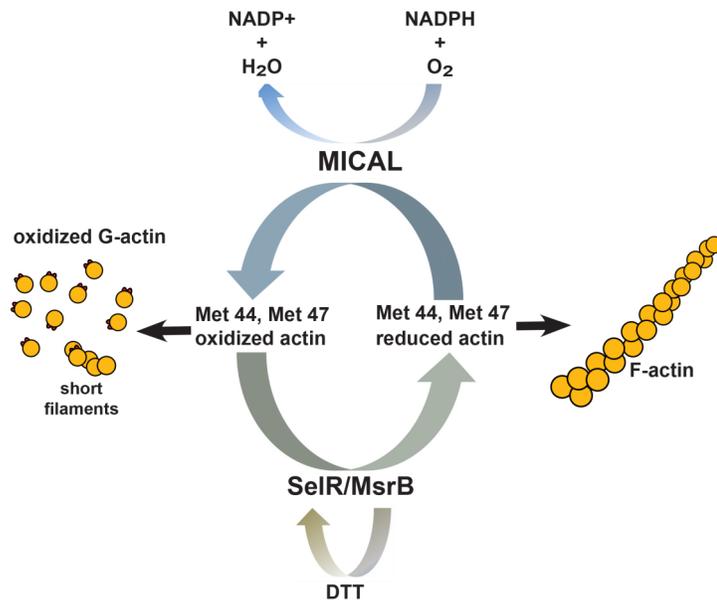
Shipped at ambient temperature. The lyophilized protein can be stored desiccated at 4°C for 6 months. For reconstitution, the product tube should be briefly centrifuged to collect the powder to the bottom of the tube.

Each tube of MB201 contains 50 µg of protein. When reconstituted with 50 µl of water, the final buffer composition is 10 mM Tris pH 8.0, 50 mM NaCl, 2.5% sucrose, and 0.5% dextran. We recommend that you make 10 µl aliquots, flash freeze, and store at -70°C.

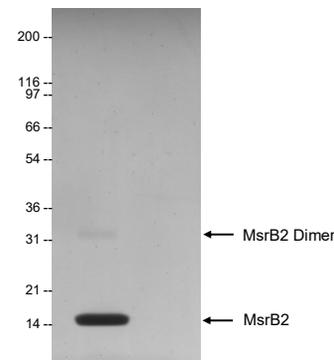
When stored and reconstituted as described, MB201 is stable for 6 months at -70°C.

### Purity

Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 4-20% gradient tris-glycine gel. MsrB2 protein was determined to be > 90% pure (Figure 2).



**Figure 1. Actin Met 44 and Met 47 physiological redox system.** A schematic diagram of the enzymes that control the physiological and reversible oxidation and reduction of methionines 44 and 47 of actin. Specifically, the MICAL family of proteins regulate oxidation of actin at Met 44 and Met 47. Conversely, the SelR/MsrB family of methionine sulfoxide reductase family specifically reduces actin at Met 44 and Met 47.



**Figure 2. MsrB2 Protein Purity Determination.** A 10 µg sample of MsrB2 protein was separated by electrophoresis in a 4-20% tris-glycine gel and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein Assay Reagent (Cat. # ADV02). Mark12 standard molecular weight markers are from Invitrogen.

### Activity

Effective MsrB2 (MB201) + DTT reduction of MICAL-oxidized actin will enhance subtilisin A cleavage of MICAL-oxidized actin (Cat. # MXA95) at M47/G48 by > 90%. See the next page for details. Whereas DTT alone will not affect subtilisin cleavage.

## Evaluating MsrB2 Activity: Subtilisin A Limited Proteolysis Application

### Rationale:

MsrB2 (MB201) is a protein that can reduce actin at amino acids M44 and M47 ( $\beta$ -actin nomenclature)<sup>[2,3]</sup>. The MsrB2 becomes oxidized during the reduction of actin and can be regenerated through reduction by DTT *in vitro*. It has been reported that M44 oxidation by MICAL is concomitant with M47 oxidation<sup>[1]</sup>. Subtilisin A is a protease that has been shown to specifically cleave actin between M47 and G48<sup>[6,7]</sup>. When actin is oxidized at M47 by MICAL the efficiency of subtilisin A cleavage is significantly reduced; conversely, when actin is reduced at M47 by MsrB2 (+DTT) the efficiency of subtilisin A cleavage should be significantly enhanced<sup>[6]</sup>. Therefore, subtilisin A can be used in limited proteolysis assays to determine MsrB2 activity, and efficiency of M47 and M44 reduction by evaluating cleaved vs uncleaved actin.

### Expected Results:

Effective MsrB2 (MB201) + DTT reduction of MICAL-oxidized actin will enhance subtilisin A cleavage of MICAL-oxidized actin (Cat. # MXA95) at M47/G48 by > 90% under the conditions described. Whereas DTT alone will not affect subtilisin cleavage.

### Reagents Needed:

MsrB2 (Cat. # MB201): When used as described below, 50  $\mu$ g will reduce 10 ml of actin at 0.5 mg/ml  
MICAL-oxidized rabbit skeletal muscle actin (Cat. # MXA95)  
G-buffer (Cat. # BSA01)  
ATP (Cat. # BSA04)  
DTT 1 M stock  
Precision red advanced protein assay (Cat. # ADV02)  
Phenylmethylsulfonyl fluoride (PMSF)  
Subtilisin A (Cat. # P5380; Sigma)

### Method:

#### Day 1

- Resuspend MICAL-oxidized rabbit skeletal muscle actin (Cat. # MXA95) to 0.2 mg/ml with G-buffer (5 mM Tris pH 8.0, 0.2 mM CaCl<sub>2</sub>, 0.2 mM ATP, 0.5 mM DTT)
- Incubate the oxidized actin on ice for 1 h
- Centrifuge the oxidized actin in a microcentrifuge at 14,000 rpm for 30 min at 4°C
- Transfer the supernatant to a new tube on ice
- Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
- Dilute oxidized actin to 0.1 mg/ml with G-buffer
- Resuspend one tube of MB201 with 50  $\mu$ l of water. The approximate concentration will be 1  $\mu$ g/ $\mu$ l (58.82  $\mu$ M). This is your stock tube
- Prepare DTT to 1 M stock
- Prepare the following 4 samples in 1.5 ml centrifuge tubes as shown in Table 1
- Incubate samples overnight at room temperature. This will allow MsrB2 to reduce the oxidized actin

Table 1: Composition of Experimental Samples for MsrB2 Reduction

sample	Name	MXA95 11.9 $\mu$ M	DTT 1 M stock	MB201 58.82 $\mu$ M stock
1	MXA95	200 $\mu$ l	0	0
2	MXA95 + DTT	200 $\mu$ l	10 mM Final	0
3	MXA95 + MB201	200 $\mu$ l	0	0.3 $\mu$ M Final
4	MXA95 + DTT + MB201	200 $\mu$ l	10 mM Final	0.3 $\mu$ M Final

Table 2: Composition of Experimental Samples for Subtilisin Digestion

Sample	Name	Add Subtilisin	Add PMSF
1	MXA95	No	Yes
2	MXA95	Yes	Yes
3	MXA95 + DTT	No	Yes
4	MXA95 + DTT	Yes	Yes
5	MXA95 + MB201	No	Yes
6	MXA95 + MB201	Yes	Yes
7	MXA95 + Both	No	Yes
8	MXA95 + Both	Yes	Yes

#### Day 2

- Aliquot 20  $\mu$ l (2  $\mu$ g actin) of each sample into individual 1.5 ml tubes. 2 aliquots per sample are needed for untreated versus subtilisin treated conditions. You should have the following 8 conditions as shown in Table 2
- Before beginning subtilisin treatment boil a beaker of water. The water should be boiling when the subtilisin reaction is complete to inactivate the protease
- Make a stock of subtilisin: 1 mg of subtilisin can be dissolved in 32 ml of 2 mM Tris pH 8.0/0.2 mM CaCl<sub>2</sub> to make a 31.25  $\mu$ g/ml stock. Aliquot and store at -70°C.
- Dilute a tube of subtilisin stock to 2  $\mu$ g/ml with G-buffer.  
(Subtilisin analysis: 1:200 w/w subtilisin to actin = 10 ng subtilisin / 2ug actin).
- Add 5  $\mu$ l (10 ng) of subtilisin to treated condition for all 4 samples  
(Subtilisin should be diluted and added to sample in less than 10 min)
- Treat actin samples with subtilisin for 15 min
- During treatment make PMSF (10 mM: 1.74 mg PMSF per ml of isopropanol).
- After 15 min of subtilisin treatment, stop the reactions by adding 1  $\mu$ l of PMSF to each sample (1-8)
- Add 5  $\mu$ l of 5x reducing sample buffer to each sample (1-8)  
**NOTE: Immediately boil samples once reducing buffer is added, as denaturing the actin sample allows trace activity of subtilisin A to rapidly cleave actin.**
- Immediately place samples into boiling water. Leave samples for 5 min
- Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes
- Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
- Visualize with Coomassie staining. See Figure 3

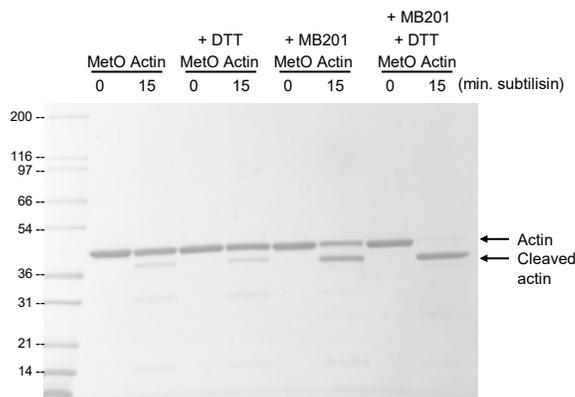


Figure 3. Subtilisin Assay on MsrB2 Treated Oxidized Actin. MICAL-oxidized actin (Cat. # MXA95) was diluted to 0.1 mg/ml (2.3  $\mu$ M) and left untreated, or treated with DTT (10 mM), MB201 (0.3  $\mu$ M), or a combination of both (see method). 2  $\mu$ g of each sample was then left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

## Using MsrB2 as a research tool: Oxidized and Reduced Actin Polymerization Application

### Rationale:

MsrB2 (MB201) is a protein that can reduce actin at amino acids M44 and M47 ( $\beta$ -actin nomenclature) [2,3]. When actin is oxidized at M44 its ability to polymerize is significantly diminished [1]; conversely when oxidized actin is reduced at M44 its ability to polymerize is restored [2,3]. Actin polymerization assays can be used to measure the polymerization capabilities of MICAL-oxidized actin vs MsrB2-reduced actin.

### Expected Results:

Effective MsrB2 (MB201) + DTT reduction of MICAL-oxidized pyrene-labeled actin (Cat. # MXAP95) will restore fluorescence signal at 1 hour to a level comparable to native pyrene-labeled actin (Cat. # AP05) when actin polymerization is performed at 0.05 mg/ml of pyrene-labeled actin. Whereas DTT alone will not restore polymerization competence.

### Reagents Needed:

MsrB2 (Cat. # MB201): When used as described below, 50  $\mu$ g is sufficient for 4900  $\mu$ l of 2x pol buffer + DTT + MB201 0.3  $\mu$ M assay buffer (Table 6), enough for 39 rxns.  
 Pyrene-labeled rabbit skeletal muscle actin (Cat. # AP05)  
 MICAL-oxidized pyrene-labeled rabbit skeletal muscle actin (Cat. # MXAP95)  
 G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl<sub>2</sub>)  
 Precision red advanced protein assay (Cat. # ADV02)  
 DTT 1 M stock  
 2x polymerization buffer (5 mM Tris pH 7.5, 4 mM MgCl<sub>2</sub>, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)

### Equipment Needed:

1. Fluorescence spectrophotometer with an excitation wavelength of 360 +/- 20 nm and an emission wavelength of 405 +/- 10 nm or 420 +/- 20 nm. Cytoskeleton recommends the SPECTRAFluor Plus from TECAN Austria GmbH or Gemini from Molecular Devices Inc.
2. Black polystyrene 96 well assay plate (Costar, Cat. # 3915).

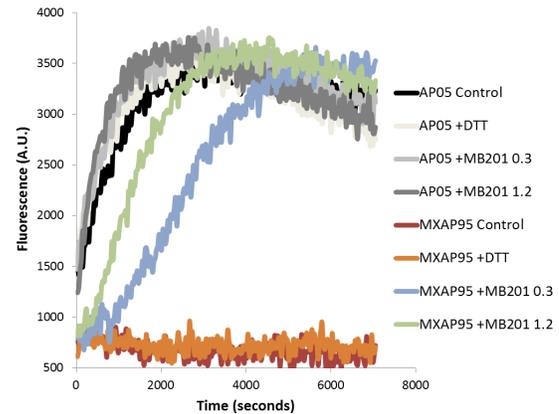
### Method:

1. Dilute both pyrene-labeled rabbit skeletal muscle actin (Cat. #AP05) and MICAL-oxidized pyrene-labeled rabbit skeletal muscle actin (Cat. #MXAP95) to 0.45 mg/ml with G-buffer (Cat. # BSA01) supplemented with 0.2 mM ATP and 1 mM DTT.
2. Leave on ice for 1 h to depolymerize actin oligomers
3. Centrifuge at 14,000 rpm at 4°C for 30 min to remove nucleating centers
4. Determine the protein concentration
5. Dilute AP05 and MXAP95 to 0.1 mg/ml with G-buffer
5. Resuspend one tube of MB201 with 50  $\mu$ l of water. The approximate concentration will be 1  $\mu$ g/ $\mu$ l (58.82  $\mu$ M). This is your stock tube
6. Prepare DTT to 1 M stock
7. Prepare the following Assay Polymerization buffers as shown in Table 6
8. Pipet 100  $\mu$ l of G-buffer into two wells (A1-2) (control samples)
9. Pipet 100  $\mu$ l of 0.1 mg/ml AP05 into eight wells (A3-10) of a black 96 well plate
10. Pipet 100  $\mu$ l of 0.1 mg/ml MXAP95 into eight wells (B3-10) of a black 96 well plate
11. Place the 96 well plate into the fluorescent spectrophotometer and read the samples for 3 min to establish a baseline fluorescent measurement
12. After 3 min add 100  $\mu$ l of Assay Polymerization Buffers into assigned wells (see Table 6)
13. Return the plate to the spectrophotometer and read the fluorescence every 30 s for 2 h

Table 6: Composition of Assay Polymerization Buffers

	2X polymerization Buffer	DTT 1 M Stock	MB201 58.82 $\mu$ M Stock	Add to Lanes: (step 12)
2x pol buffer	1 ml	0	0	A1-4 B1-4
2x pol buffer + DTT	500 $\mu$ l	20 mM	0	A5-6 B5-6
2x pol buffer + DTT + MB201 0.3 $\mu$ M	500 $\mu$ l	20 mM	0.6 $\mu$ M	A7-8 B7-8
2x pol buffer + DTT + MB201 1.2 $\mu$ M	500 $\mu$ l	20 mM	2.4 $\mu$ M	A9-10 B9-10

14. A typical polymerization fluorescent enhancement curve is shown in Figure 5



**Figure 5. Oxidized Actin Polymerization +/- MsrB2 Treatment.** Pyrene-labeled actin (Cat. # AP05) and MICAL-oxidized pyrene labeled actin (Cat. # MXAP95) were diluted to 0.1 mg/ml (2.3  $\mu$ M) (see method). Samples were then incubated with 2x polymerization buffer supplemented with nothing (labeled Control), DTT alone (labeled +DTT), MB201 0.3  $\mu$ M + DTT (labeled +MB201 0.3), or MB201 1.2  $\mu$ M + DTT (labeled +MB201 1.2). Upon actin polymerization fluorescence was detected with a spectrophotometer (see method below). A.U. = arbitrary units

### References

1. Hung RJ, Pak CW, Terman JR. Direct redox regulation of F-actin assembly and disassembly by MICAL. *Science*. 2011;334(6063):1710-3. 10.1126/science.1211956.
2. Kaya A, Lee BC, Gladyshev VN. Regulation of protein function by reversible methionine oxidation and the role of selenoprotein MsrB1. *Antioxid Redox Signal*. 2015;23(10):814-22. 10.1089/ars.2015.6385.
3. Hung RJ, Spaeth CS, Yesilyurt HG, Terman JR. SelR reverses Mical-mediated oxidation of actin to regulate F-actin dynamics. *Nat Cell Biol*. 2013;15(12):1445-54. 10.1038/ncb2871.
4. Lee BC, Peterfi Z, Hoffmann FW, Moore RE, Kaya A, Avanesov A, et al. MsrB1 and MICALs regulate actin assembly and macrophage function via reversible stereoselective methionine oxidation. *Mol Cell*. 2013;51(3):397-404. 10.1016/j.molcel.2013.06.019.
5. Binger KJ, Griffin MD, Heinemann SH, Howlett GJ. Methionine-oxidized amyloid fibrils are poor substrates for human methionine sulfoxide reductases A and B2. *Biochemistry*. 2010;49(14):2981-3. 10.1021/bi902203m.
6. Grintsevich EE, Yesilyurt HG, Rich SK, Hung RJ, Terman JR, Reisler E. F-actin dismantling through a redox-driven synergy between Mical and cofilin. *Nat Cell Biol*. 2016;18(8):876-85. 10.1038/ncb3390.
7. Schwyter D, Phillips M, Reisler E. Subtilisin-cleaved actin: polymerization and interaction with myosin subfragment 1. *Biochemistry*. 1989; 28:5889-5895.

### Product Citations/Related Products

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## 6xHis MsrB2 Protein aa: 42-182

(Human recombinant)

Cat. # MB201-XL

Lot:

Upon arrival, store at 4°C (desiccated)

See datasheet for storage after reconstitution

<b>Form:</b>	Lyophilized white powder
<b>Amount of material:</b>	1 x 1 mg
<b>Validated applications:</b>	Actin Reduction at Met 44, Met 47

### Background Information

Recently, it was shown that the MICAL protein can interact with F-actin to promote oxidation at Met44 and Met47<sup>[1]</sup> (see Figure 1). Subsequent studies discovered that MICAL-mediated methionine sulfoxide (MetO) of actin is reversed by the SelR/MsrB family of methionine sulfoxide reductase (Msr) enzymes<sup>[2,3]</sup>. The Msr family are well conserved proteins that function to reduce oxidized methionine to L-methionine<sup>[4]</sup>.

Two groups independently identified SelR (MsrB) as the enzyme responsible for reduction of the Met 44 and Met47 amino acids of actin; importantly, this reduction of Met44 and Met47 was sufficient to restore normal actin polymerization<sup>[2,3]</sup>. These studies determined that the CxxS catalytic cysteine motif was essential for MsrB effects, as mutating the cysteine residue abolished MsrB's ability to restore actin polymerization<sup>[2]</sup>. These studies also determined that SelR/MsrB selectively reduced MICAL-oxidized actin, while another methionine sulfoxide reductase member, MsrA, did not<sup>[2,3]</sup>. Since SelR/MsrB specifically reduces R-isomer (MetO) and MsrA specifically reduces S-isomer MetO the groups concluded that MICAL stereo-specifically oxidized actin with MetO R-isomer. Collectively, these data describe a reversible, specific redox system that controls actin dynamics and cytoskeletal organization through regulation of a specific MetO of actin.

### Material

Human MSRB2 protein (MB201) [accession # Q9Y3D2 (MSRB2\_HUMAN)] has been produced and purified from a bacterial expression system. The recombinant protein is N-terminally 6x Histidine tagged and is comprised of amino acids 42-182<sup>[5]</sup>. MB201 has an approximate molecular weight of 17 kDa. MB201 is supplied as a white lyophilized powder.

### Storage and Reconstitution

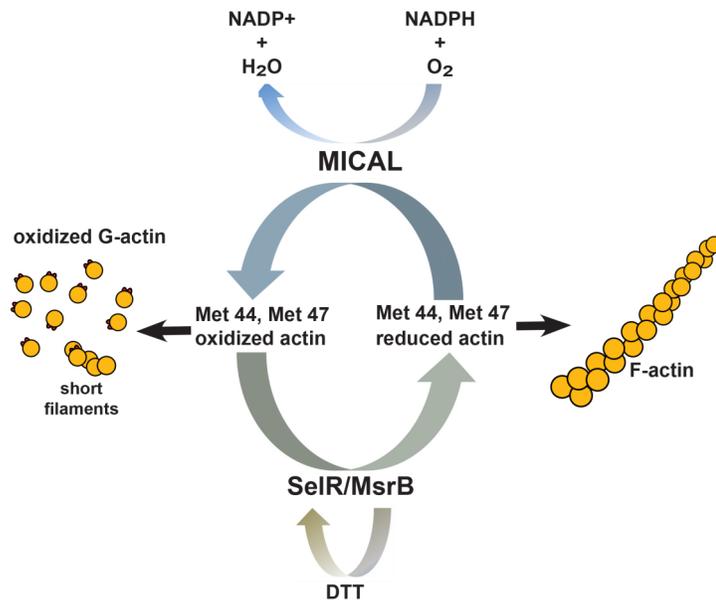
Shipped at ambient temperature. The lyophilized protein can be stored desiccated at 4°C for 6 months. For reconstitution, the product tube should be briefly centrifuged to collect the powder to the bottom of the tube.

Each tube of MB201 contains 1 x 1 mg of protein. When reconstituted with 1 ml of water, the final buffer composition is 10 mM Tris pH 8.0, 50 mM NaCl, 2.5% sucrose, and 0.5% dextran. We recommend that you make 10 µl aliquots, flash freeze, and store at -70°C.

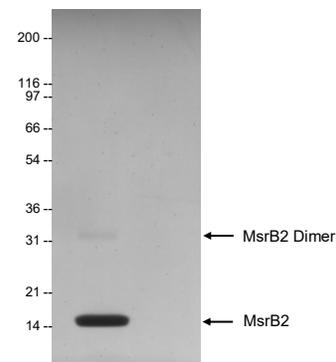
When stored and reconstituted as described, MB201 is stable for 6 months at -70°C.

### Purity

Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 4-20% gradient tris-glycine gel. MsrB2 protein was determined to be > 90% pure (Figure 2).



**Figure 1. Actin Met 44 and Met 47 physiological redox system.** A schematic diagram of the enzymes that control the physiological and reversible oxidation and reduction of methionines 44 and 47 of actin. Specifically, the MICAL family of proteins regulate oxidation of actin at Met 44 and Met 47. Conversely, the SelR/MsrB family of methionine sulfoxide reductase family specifically reduces actin at Met 44 and Met 47.



**Figure 2. MsrB2 Protein Purity Determination.** A 10 µg sample of MsrB2 protein was separated by electrophoresis in a 4-20% tris-glycine gel and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein Assay Reagent (Cat. # ADV02). Mark12 standard molecular weight markers are from Invitrogen.

### Activity

Essential MsrB2 (MB201) + DTT reduction of MICAL-oxidized actin will enhance subtilisin A cleavage of MICAL-oxidized actin (Cat. # MXA95) at M47/G48 by > 90%. See the next page for details. Whereas DTT alone will not affect subtilisin cleavage.

## Evaluating MsrB2 Activity: Subtilisin A Limited Proteolysis Application

### Rationale:

MsrB2 (MB201) is a protein that can reduce actin at amino acids M44 and M47 ( $\beta$ -actin nomenclature)<sup>[2,3]</sup>. The MsrB2 becomes oxidized during the reduction of actin and can be regenerated through reduction by DTT *in vitro*. It has been reported that M44 oxidation by MICAL is concomitant with M47 oxidation<sup>[1]</sup>. Subtilisin A is a protease that has been shown to specifically cleave actin between M47 and G48<sup>[6,7]</sup>. When actin is oxidized at M47 by MICAL the efficiency of subtilisin A cleavage is significantly reduced; conversely, when actin is reduced at M47 by MsrB2 (+DTT) the efficiency of subtilisin A cleavage should be significantly enhanced<sup>[6]</sup>. Therefore, subtilisin A can be used in limited proteolysis assays to determine MsrB2 activity, and efficiency of M47 and M44 reduction by evaluating cleaved vs uncleaved actin.

### Expected Results:

Effective MsrB2 (MB201) + DTT reduction of MICAL-oxidized actin will enhance subtilisin A cleavage of MICAL-oxidized actin (Cat. # MXA95) at M47/G48 by > 90% under the conditions described. Whereas DTT alone will not affect subtilisin cleavage.

### Reagents Needed:

MsrB2 (Cat. # MB201): When used as described below, 1 mg will reduce 200 ml of actin at 0.5 mg/ml  
MICAL-oxidized rabbit skeletal muscle actin (Cat. # MXA95)  
G-buffer (Cat. # BSA01)  
ATP (Cat. # BSA04)  
DTT 1 M stock  
Precision red advanced protein assay (Cat. # ADV02)  
Phenylmethylsulfonyl fluoride (PMSF)  
Subtilisin A (Cat. # P5380; Sigma)

### Method:

#### Day 1

- Resuspend MICAL-oxidized rabbit skeletal muscle actin (Cat. # MXA95) to 0.2 mg/ml with G-buffer (5 mM Tris pH 8.0, 0.2 mM CaCl<sub>2</sub>, 0.2 mM ATP, 0.5 mM DTT)
- Incubate the oxidized actin on ice for 1 h
- Centrifuge the oxidized actin in a microcentrifuge at 14,000 rpm for 30 min at 4°C
- Transfer the supernatant to a new tube on ice
- Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
- Dilute oxidized actin to 0.1 mg/ml with G-buffer
- Resuspend one tube of MB201 with 50  $\mu$ l of water. The approximate concentration will be 1  $\mu$ g/ $\mu$ l (58.82  $\mu$ M). This is your stock tube
- Prepare DTT to 1 M stock
- Prepare the following 4 samples in 1.5 ml centrifuge tubes as shown in Table 1
- Incubate samples overnight at room temperature. This will allow MsrB2 to reduce the oxidized actin

Table 1: Composition of Experimental Samples for MsrB2 Reduction

sample	Name	MXA95 11.9 $\mu$ M	DTT 1 M stock	MB201 58.82 $\mu$ M stock
1	MXA95	200 $\mu$ l	0	0
2	MXA95 + DTT	200 $\mu$ l	10 mM Final	0
3	MXA95 + MB201	200 $\mu$ l	0	0.3 $\mu$ M Final
4	MXA95 + DTT + MB201	200 $\mu$ l	10 mM Final	0.3 $\mu$ M Final

Table 2: Composition of Experimental Samples for Subtilisin Digestion

Sample	Name	Add Subtilisin	Add PMSF
1	MXA95	No	Yes
2	MXA95	Yes	Yes
3	MXA95 + DTT	No	Yes
4	MXA95 + DTT	Yes	Yes
5	MXA95 + MB201	No	Yes
6	MXA95 + MB201	Yes	Yes
7	MXA95 + Both	No	Yes
8	MXA95 + Both	Yes	Yes

### Day 2

- Aliquot 20  $\mu$ l (2  $\mu$ g actin) of each sample into individual 1.5 ml tubes. 2 aliquots per sample are needed for untreated versus subtilisin treated conditions. You should have the following 8 conditions as shown in Table 2
- Before beginning subtilisin treatment boil a beaker of water. The water should be boiling when the subtilisin reaction is complete to inactivate the protease
- Make a stock of subtilisin: 1 mg of subtilisin can be dissolved in 32 ml of 2 mM Tris pH 8.0/0.2 mM CaCl<sub>2</sub> to make a 31.25  $\mu$ g/ml stock. Aliquot and store at -70°C.
- Dilute a tube of subtilisin stock to 2  $\mu$ g/ml with G-buffer.  
(Subtilisin analysis: 1:200 w/w subtilisin to actin = 10 ng subtilisin / 2ug actin).
- Add 5  $\mu$ l (10 ng) of subtilisin to treated condition for all 4 samples  
(Subtilisin should be diluted and added to sample in less than 10 min)
- Treat actin samples with subtilisin for 15 min
- During treatment make PMSF (10 mM: 1.74 mg PMSF per ml of isopropanol).
- After 15 min of subtilisin treatment, stop the reactions by adding 1  $\mu$ l of PMSF to each sample (1-8)
- Add 5  $\mu$ l of 5x reducing sample buffer to each sample (1-8)  
**NOTE: Immediately boil samples once reducing buffer is added, as denaturing the actin sample allows trace activity of subtilisin A to rapidly cleave actin.**
- Immediately place samples into boiling water. Leave samples for 5 min
- Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes
- Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
- Visualize with Coomassie staining. See Figure 3

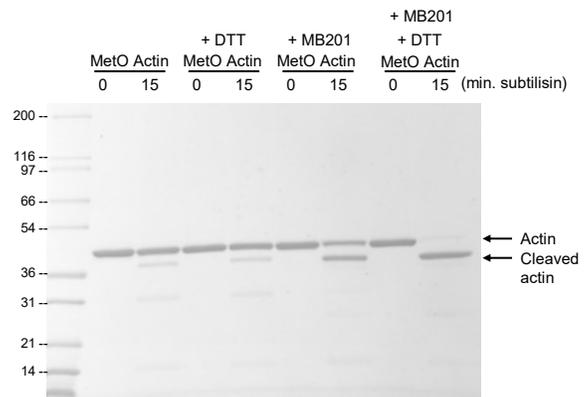


Figure 3. Subtilisin Assay on MsrB2 Treated Oxidized Actin. MICAL-oxidized actin (Cat. # MXA95) was diluted to 0.1 mg/ml (2.3  $\mu$ M) and left untreated, or treated with DTT (10 mM), MB201 (0.3  $\mu$ M), or a combination of both (see method). 2  $\mu$ g of each sample was then left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

## Using MsrB2 as a research tool: Oxidized and Reduced Actin Polymerization Application

### Rationale:

MsrB2 (MB201) is a protein that can reduce actin at amino acids M44 and M47 ( $\beta$ -actin nomenclature) [2,3]. When actin is oxidized at M44 its ability to polymerize is significantly diminished [1]; conversely when oxidized actin is reduced at M44 its ability to polymerize is restored [2,3]. Actin polymerization assays can be used to measure the polymerization capabilities of MICAL-oxidized actin vs MsrB2-reduced actin.

### Expected Results:

Effective MsrB2 (MB201) + DTT reduction of MICAL-oxidized pyrene-labeled actin (Cat. # MXAP95) will restore fluorescence signal at 1 hour to a level comparable to native pyrene-labeled actin (Cat. # AP05) when actin polymerization is performed at 0.05 mg/ml of pyrene-labeled actin. Whereas DTT alone will not restore polymerization competence.

### Reagents Needed:

MsrB2 (Cat. # MB201): When used as described below, 1 mg is sufficient for 98 ml of 2x pol buffer + DTT + MB201 0.3  $\mu$ M assay buffer (Table 6), enough for 784 rxns.

Pyrene-labeled rabbit skeletal muscle actin (Cat. # AP05)  
MICAL-oxidized pyrene-labeled rabbit skeletal muscle actin (Cat. # MXAP95)  
G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl<sub>2</sub>)  
Precision red advanced protein assay (Cat. # ADV02)  
DTT 1 M stock

2x polymerization buffer (5 mM Tris pH 7.5, 4 mM MgCl<sub>2</sub>, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)

### Equipment Needed:

- Fluorescence spectrophotometer with an excitation wavelength of 360 +/- 20 nm and an emission wavelength of 405 +/- 10 nm or 420 +/- 20 nm. Cytoskeleton recommends the SPECTRAFluor Plus from TECAN Austria GmbH or Gemini from Molecular Devices Inc.
- Black polystyrene 96 well assay plate (Costar, Cat. # 3915).

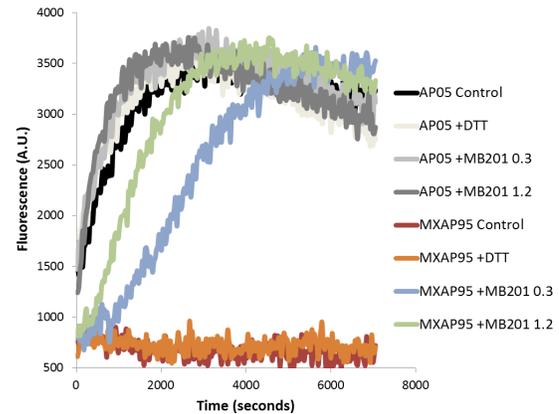
### Method:

- Dilute both pyrene-labeled rabbit skeletal muscle actin (Cat. #AP05) and MICAL-oxidized pyrene-labeled rabbit skeletal muscle actin (Cat. #MXAP95) to 0.45 mg/ml with G-buffer (Cat. # BSA01) supplemented with 0.2 mM ATP and 1 mM DTT.
- Leave on ice for 1 h to depolymerize actin oligomers
- Centrifuge at 14,000 rpm at 4°C for 30 min to remove nucleating centers
- Determine the protein concentration
- Dilute AP05 and MXAP95 to 0.1 mg/ml with G-buffer
- Resuspend one tube of MB201 with 50  $\mu$ l of water. The approximate concentration will be 1  $\mu$ g/ $\mu$ l (58.82  $\mu$ M). This is your stock tube
- Prepare DTT to 1 M stock
- Prepare the following Assay Polymerization buffers as shown in Table 6
- Pipet 100  $\mu$ l of G-buffer into two wells (A1-2) (control samples)
- Pipet 100  $\mu$ l of 0.1 mg/ml AP05 into eight wells (A3-10) of a black 96 well plate
- Pipet 100  $\mu$ l of 0.1 mg/ml MXAP95 into eight wells (B3-10) of a black 96 well plate
- Place the 96 well plate into the fluorescent spectrophotometer and read the samples for 3 min to establish a baseline fluorescent measurement
- After 3 min add 100  $\mu$ l of Assay Polymerization Buffers into assigned wells (see Table 6)
- Return the plate to the spectrophotometer and read the fluorescence every 30 s for 2 h

Table 6: Composition of Assay Polymerization Buffers

	2X polymerization Buffer	DTT 1 M Stock	MB201 58.82 $\mu$ M Stock	Add to Lanes: (step 12)
2x pol buffer	1 ml	0	0	A1-4 B1-4
2x pol buffer + DTT	500 $\mu$ l	20 mM	0	A5-6 B5-6
2x pol buffer + DTT + MB201 0.3 $\mu$ M	500 $\mu$ l	20 mM	0.6 $\mu$ M	A7-8 B7-8
2x pol buffer + DTT + MB201 1.2 $\mu$ M	500 $\mu$ l	20 mM	2.4 $\mu$ M	A9-10 B9-10

14. A typical polymerization fluorescent enhancement curve is shown in Figure 5



**Figure 5. Oxidized Actin Polymerization +/- MsrB2 Treatment.** Pyrene-labeled actin (Cat. # AP05) and MICAL-oxidized pyrene labeled actin (Cat. # MXAP95) were diluted to 0.1 mg/ml (2.3  $\mu$ M) (see method). Samples were then incubated with 2x polymerization buffer supplemented with nothing (labeled Control), DTT alone (labeled +DTT), MB201 0.3  $\mu$ M + DTT (labeled +MB201 0.3), or MB201 1.2  $\mu$ M + DTT (labeled +MB201 1.2). Upon actin polymerization fluorescence was detected with a spectrophotometer (see method below). A.U. = arbitrary units

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