V. 1.2

Actin Protein (MICAL-Oxidized): Rabbit Skeletal Muscle

(Rabbit Muscle, > 95% Pure)
Cat. # MXA95
Lot:
Upon arrival, store at 4°C (desiccated)
See datasheet for storage after reconstitution

Form:	Lyophilized white powder
Amount of material:	2 x 250 µg
Validated applications:	Sedimentation assay

Online Datasheet Contains
MXA95 (V1.2) and MXA95-XL (V1.2)

Background Information

MICAL is an intracellular flavoprotein monoxygenase, conserved from insects to mammals, that functions as a catalyst for oxidation-reduction (redox) reactions [1,2]. Terman's group showed that MICAL interacts with F-actin and uses NADPH as a cofactor to oxidize actin at Met44 and Met47 [3] (see Figure 1). Importantly, MICAL-mediated effects on actin were not occurring through a diffusible oxidant like H_2O_2, as reductants like DTT did not alter MICAL activity, and close proximity between MICAL and actin were necessary for oxidation [3]. MICAL oxidation is both physiological and enzymatically reversible by the MsrB family of methionine sulfoxide reductases [4,5].

Functionally, oxidation of Met44 has a profound effect on actin polymerization because the residue resides in the D-loop of subdomain 2 of the protein, which is critical for actin subunit contacts; thus, upon oxidation, Met44 becomes negatively charged and interferes with actin monomer-monomer interaction and promotes F-actin severing and depolymerization [6].

Regulation of actin oxidation at Met44/Met47 has been shown to destabilize F-actin in vivo [7] and to play a key role in a growing number of cellular processes, including, cytokinesis [8], axonal guidance, dendritic organization, synaptic development, heart and muscle development and cell viability [5].

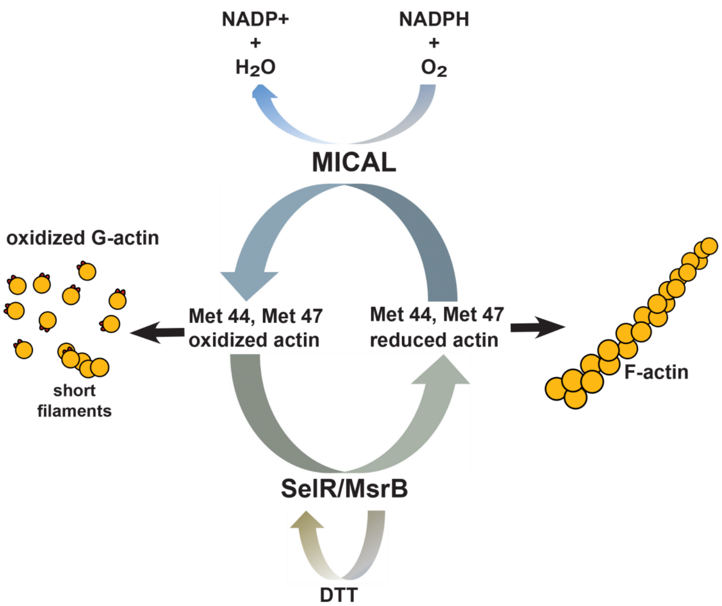

Figure 1. Actin Met 44 and Met 47 physiological redox system. A schematic diagram of the enzymes that control the physiological and reversible oxidation and reduction of methionines 44 and 47 of actin. Specifically, the MICAL family of proteins regulate oxidation of actin at Met 44 and Met 47. Conversely,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

Material

Rabbit skeletal muscle actin protein (MICAL-oxidized) (MXA95) has been enzymatically oxidized at methionines 44 and 47 (β-actin nomenclature) with the MICAL flavoprotein monoxygenase protein (cat. # MIC01). MICAL-oxidized rabbit muscle actin has an approximate molecular weight of 43 kDa and 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.

The protein should be reconstituted to 10 mg/ml with 25 µl of cold distilled water; it will then be in the following buffer: 5 mM Tris-HCl pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 5% (w/v) sucrose and 1% (w/v) dextran. The concentrated protein can then be snap frozen in liquid nitrogen and stored at - 70°C where it is stable for 6 months.

For working concentrations, further dilution of the protein should be made with General Actin Buffer (Cat. # BSA01) supplemented with 0.2 mM ATP (Cat. # BSA04) and 0.5 mM DTT. MICAL-oxidized actin is a labile protein and should be handled with care. Avoid repeated freeze-thaw cycles and do not freeze below 10 mg/ml

When stored and reconstituted as described, MXA95 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. Actin protein (MICAL-oxidized) was determined to be ≥ 95% pure (**Figure 2**).

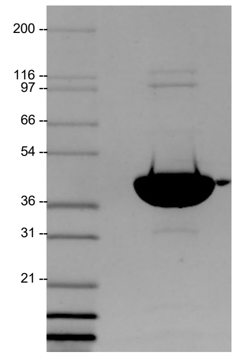

Figure 2. Actin Protein (MICAL-Oxidized) Purity Determination. A 50 µg sample of actin protein (MICAL-oxidized) was separated by electrophoresis in a 4- 20% tris-glycine gel and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein Assay Reagent (Cat. # ADV02). Mark12 standard molecular weight markers are from Invitrogen.

Activity

Purified MICAL-oxidized actin has reduced susceptibility to subtilisin A cleavage at M47/G48 by > 90%. See the next page for details.

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Evaluating Methionine Oxidation of Actin: Subtilisin A Limited Proteolysis Application

Rationale:
Subtilisin A is a protease that has been shown to cleave actin between M47 and G48 [9,10]. When actin is oxidized at M47 the efficiency of subtilisin A cleavage is significantly reduced [9]. MICAL-1 is a protein that can specifically oxidize actin at amino acids M44 and M47 (β-actin nomenclature). It has been reported that M44 oxidation by MICAL is concomitant with M47 oxidation [3]. Therefore, subtilisin A can be used in limited proteolysis assays to evaluate MICAL-oxidized actin versus native actin by measuring cleaved versus uncleaved actin.

Expected Results:
Purified MICAL-oxidized actin has reduced susceptibility to subtilisin A cleavage at M47/G48 by > 90% under the conditions described below.

Reagents Needed:
MICAL-oxidized Rabbit skeletal muscle actin (Cat. # MXA95): 250 µg is sufficient for roughly 113 subtilisin reactions when used at 2 µg / rxn
Rabbit skeletal muscle actin (Cat. # AKL99)
G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)
Precision red advanced protein assay (Cat. # ADV02)
Phenylmethylsulfonyl fluoride (PMSF)
Subtilisin A (Cat. # P5380; Sigma)

Method:
__Day 1__
1. Resuspend MICAL-oxidized rabbit skeletal muscle actin (Cat. # MXA95) and rabbit skeletal muscle actin (Cat. # AKL99) to 0.2 mg/ml with G-buffer (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0. 5 mM DTT)
2. Incubate both MXA95 and AKL99 actin on ice for 2 hours
   longer incubation is necessary for AKL99 to disperse nucleation bodies
3. Centrifuge the MXA95 and AKL99 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MXA95 and AKL99 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MXA95 and AKL99 actin samples to 0.1 mg/ml with G-buffer
7. Aliquot 20 µl (2 µg 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 4 conditions as shown in Table 1
8. Before beginning subtilisin treatment boil a beaker of water. The water should be boiling when the subtilisin reaction is complete to inactivate the protease
5. 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 CaCl2 to make a 31.25 µg/ml stock. Aliquot and store at -70ºC.
6. Dilute a tube of subtilisin stock to 2 µg/ml with G-buffer.
   (*Subtilisin analysis: 1:200 w/w subtilisin to actin = 10 ng subtilisin / 2ug actin*)
10. Add 10 ng of subtilisin to treated condition for all 4 samples
   (*Subtilisin should be diluted and added to sample __in less than 10 min__*)
11. Treat actin samples with subtilisin for 15 min
12. During treatment make PMSF (10 mM: 1.74 mg PMSF per ml of isopropanol)
13. After 15 min of subtilisin treatment, stop the reactions by adding 1 µl of PMSF to each sample (1-4)
14. Add 5 µl of 5x reducing sample buffer to each sample (1-4)
   NOTE: Immediately boil samples once reducing buffer is added, as dena turing the actin sample allows trace activity of subtilisin A to rapidly cleave actin.
15. Immediately place samples into boiling water. Leave samples for 5 min
16. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes

Table 1: Composition of Experimental Samples for Subtilisin Digestion

Sample	Name	Add Subtilisin	Add PMSF
1	ALK99	No	Yes
2	AKL99	Yes	Yes
3	MXA95	No	Yes
4	MXA95	Yes	Yes

17. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
18. Visualize with Coomassie staining. See Figure 3

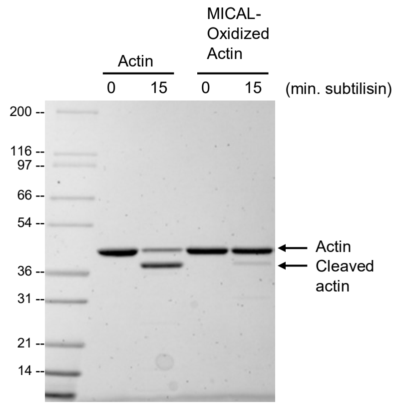

Figure 3. Subtilisin Assay on MICAL-Oxidized Actin vs Native Actin. Actin (Cat. # AKL99) and MICAL-oxidized actin (Cat. # MXA95) was diluted to 0.1 mg/ml (2.3 µM). 2 µg of each sample was then left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

Evaluating Methionine Oxidation of Actin: Sedimentation Application

Rationale:

When actin is oxidized at M44 its ability to polymerize is significantly diminished [3]. However, with increasing concentrations, oxidized actin can form augmented polymers [9]. Sedimentation assays can be used to measure the polymerization capabilities of native actin versus MICAL-oxidized actin.

Expected Results:

Purified MICAL-oxidized actin will polymerize less efficiently relative to native actin in an actin concentration dependent manner as shown in Table 2

Table 2: Expected % actin polymer formed with native versus oxidized actin

	% polymer unoxidized actin	% polymer MIC01 oxidized actin
0.1 mg/ml actin	80-90%	40-50%
0.4 mg/ml actin	85-95%	68-78%

Reagents Needed:

MICAL-oxidized Rabbit skeletal muscle actin (Cat. # MXA95): 250 µg is sufficient for 11 sedimentation reactions when used at 0.1 mg/ml and 2.5 reactions when used at 0.4 mg/ml

Rabbit skeletal muscle actin (Cat. # AKL99)

G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)

Precision red advanced protein assay (Cat. # ADV02)

2x polymerization buffer (5 mM Tris pH 7.5, 4 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)

Method:

1. Resuspend MICAL-oxidized rabbit skeletal muscle actin (Cat. # MXA95) and rabbit skeletal muscle actin (Cat. # AKL99) to 1.0 mg/ml with G-buffer (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0. 5 mM DTT)
2. Incubate both MXA95 and AKL99 actin on ice for 1 h
3. Centrifuge the MXA95 and AKL99 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MXA95 and AKL99 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MXA95 and AKL99 actin samples to the following conc. 0.8 mg/ml and 0.2 mg/ml with G-buffer
6. Prepare 2x polymerization buffer
7. Prepare four microcentrifuge tubes labeled 1-4
8. Prepare the following samples in the microcentrifuge tubes as shown in Table 3
9. Transfer 200 µl of each sample to tubes compatible for ultracentrifugation.
   Accurate loading is important for tube balance
10. Incubate at room temperature for 1 h
11. Centrifuge at 100,000 g in an ultracentrifuge for 1.5 h
12. After centrifugation remove the top 180 µl of supernatant from each sample and place into new 1.5 ml tubes labeled S1-S4
    Leave the remaining supernatant, as the pellet may become detached if disturbed

Table 3: Composition of Experimental Samples

sample	name	0.2mg/ml actin	0.8 mg/ml actin	2x pol buffer
1	AKL99 - 0.1 mg/ml	110 µl AKL99	0	110 µl
2	MXA95 - 0.1 mg/ml	110 µl MXA95	0	110 µl
3	AKL99 - 0.4 mg/ml	0	110 µl AKL99	110 µl
4	MXA95 - 0.4 mg/ml	0	110 µl MXA95	110 µl

13. Add 36 µl of 5x reducing sample buffer to tubes S1-S4
14. Resuspend pellets in 200 µl of 1x reducing sample buffer
15. Boil supernatant and pellet samples for 5 min
16. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes
17. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
    Use 40 µl of supernatant and pellet for samples 1 and 2, and 10 µl of each for samples 3 and 4 which will result in equal actin concentration loading between the 0.1 mg/ml and 0.4 mg/ml samples
18. Visualize with Coomassie staining. See Figure 4

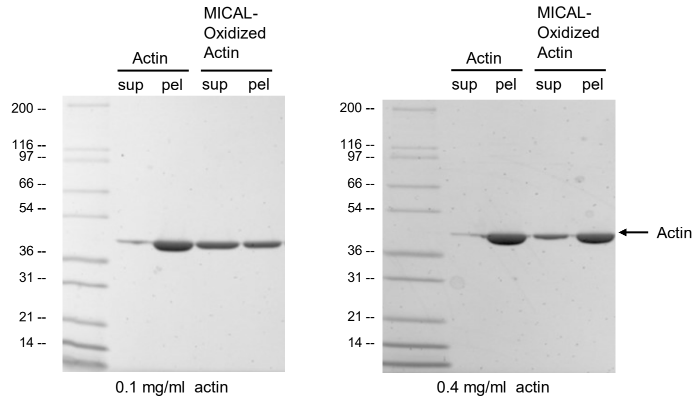

Figure 4. Actin Sedimentation Oxidized Versus Native Actin. Actin (Cat. # AKL99) and MIcal-oxidized actin was diluted to 0.2 mg/ml (4.6 µM) or 0.8 mg/ml (18.4 µM) (see method). Samples were then incubated with 2x polymerization buffer at room temperature. Samples were spun in an ultracentrifuge at 100,000 g for 1.5 h. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

References

1. Terman JR, Mao T, Pasterkamp RJ, Yu HH, Kolodkin AL. MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. *Cell*. 2002;109 (7):887-900,
2. Wu H, Yesilyurt HG, Yoon J, Terman JR. The MICALs are a Family of F-actin Dismantling Oxidoreductases Conserved from Drosophila to Humans. *Sci Rep*. 2018;8(1):937, 10.1038/s41598-017-17943-5.
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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. 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.
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10. Schwyter D, Phillips M, Reisler E. Subtilisin-cleaved actin: polymerization and interaction with myosin subfragment 1. *Biochemistry*. 1989; 28:5889–5895.

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Actin Protein (MICAL-Oxidized): Rabbit Skeletal Muscle

(Rabbit Muscle, > 95% Pure)
Cat. # MXA95-XL
Lot:
Upon arrival, store at 4°C (desiccated)
See datasheet for storage after reconstitution

Form:	Lyophilized white powder
Amount of material:	1 x 1 mg
Validated applications:	Sedimentation assay

Background Information

MICAL is an intracellular flavoprotein monoxygenase, conserved from insects to mammals, that functions as a catalyst for oxidation-reduction (redox) reactions [1,2]. Terman's group showed that MICAL interacts with F-actin and uses NADPH as a cofactor to oxidize actin at Met44 and Met47 [3] (see Figure 1). Importantly, MICAL-mediated effects on actin were not occurring through a diffusible oxidant like H_2O_2, as reductants like DTT did not alter MICAL activity, and close proximity between MICAL and actin were necessary for oxidation [3]. MICAL oxidation is both physiological and enzymatically reversible by the MsrB family of methionine sulfoxide reductases [4,5].

Functionally, oxidation of Met44 has a profound effect on actin polymerization because the residue resides in the D-loop of subdomain 2 of the protein, which is critical for actin subunit contacts; thus, upon oxidation, Met44 becomes negatively charged and interferes with actin monomer-monomer interaction and promotes F-actin severing and depolymerization [6].

Regulation of actin oxidation at Met44/Met47 has been shown to destabilize F-actin in vivo [7] and to play a key role in a growing number of cellular processes, including, cytokinesis [8], axonal guidance, dendritic organization, synaptic development, heart and muscle development and cell viability [5].

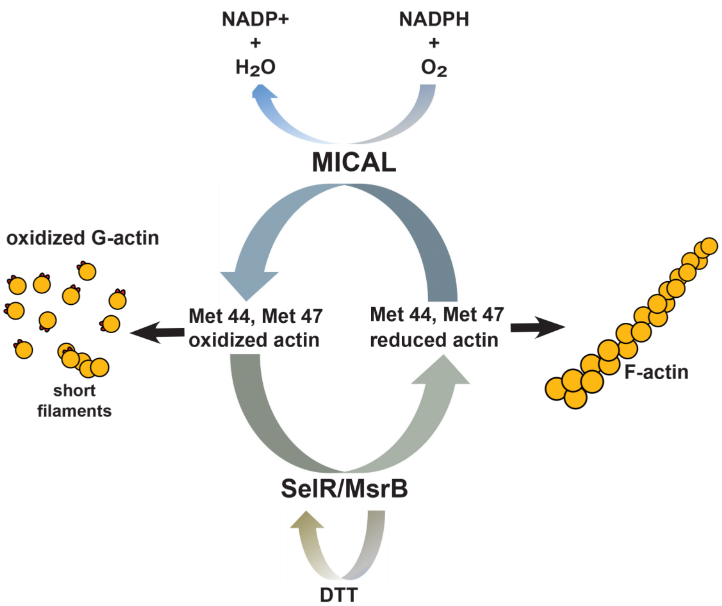

Figure 1. Actin Met 44 and Met 47 physiological redox system. A schematic diagram of the enzymes that control the physiological and reversible oxidation and reduction of methionines 44 and 47 of actin. Specifically, the MICAL family of proteins regulate oxidation of actin at Met 44 and Met 47. Conversely,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

Material

Rabbit skeletal muscle actin protein (MICAL-oxidized) (MXA95) has been enzymatically oxidized at methionines 44 and 47 (β-actin nomenclature) with the MICAL flavoprotein monoxygenase protein Cat. # MIC01). MICAL-oxidized rabbit muscle actin has an approximate molecular weight of 43 kDa and 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.

The protein should be reconstituted to 10 mg/ml with 100 µl of cold distilled water; it will then be in the following buffer: 5 mM Tris-HCl pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 5% (w/v) sucrose and 1% (w/v) dextran. The concentrated protein can then be snap frozen in liquid nitrogen and stored at - 70°C where it is stable for 6 months.

For working concentrations, further dilution of the protein should be made with General Actin Buffer (Cat. # BSA01) supplemented with 0.2 mM ATP (Cat. # BSA04) and 0.5 mM DTT. MICAL-oxidized actin is a labile protein and should be handled with care. Avoid repeated freeze-thaw cycles and do not freeze below 10 mg/ml

When stored and reconstituted as described, MXA95 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. Actin protein (MICAL-oxidized) was determined to be ≥ 95% pure (**Figure 2**).

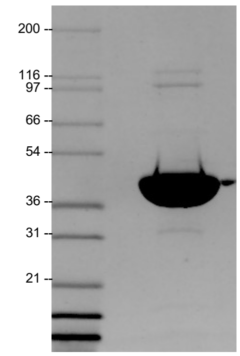

Figure 2. Actin Protein (MICAL-Oxidized) Purity Determination. A 50 µg sample of actin protein (MICAL-oxidized) was separated by electrophoresis in a 4- 20% tris-glycine gel and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein Assay Reagent (Cat. # ADV02). Mark12 standard molecular weight markers are from Invitrogen.

Activity

Purified MICAL-oxidized actin has reduced susceptibility to subtilisin A cleavage at M47/G48 by > 90%. See the next page for details.

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Evaluating Methionine Oxidation of Actin: Subtilisin A Limited Proteolysis Application

Rationale:
Subtilisin A is a protease that has been shown to cleave actin between M47 and G48 [9,10]. When actin is oxidized at M47 the efficiency of subtilisin A cleavage is significantly reduced [9]. MICAL-1 is a protein that can specifically oxidize actin at amino acids M44 and M47 (β-actin nomenclature). It has been reported that M44 oxidation by MICAL is concomitant with M47 oxidation [3]. Therefore, subtilisin A can be used in limited proteolysis assays to evaluate MICAL-oxidized actin versus native actin by measuring cleaved versus uncleaved actin.

Expected Results:
Purified MICAL-oxidized actin has reduced susceptibility to subtilisin A cleavage at M47/G48 by > 90% under the conditions described below.

Reagents Needed:
MICAL-oxidized Rabbit skeletal muscle actin (Cat. # MXA95): 1 mg is sufficient for roughly 454 subtilisin reactions when used at 2 µg / rxn
Rabbit skeletal muscle actin (Cat. # AKL99)
G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)
Precision red advanced protein assay (Cat. # ADV02)
Phenylmethylsulfonyl fluoride (PMSF)
Subtilisin A (Cat. # P5380; Sigma)

Method:
Day 1

1. Resuspend MICAL-oxidized rabbit skeletal muscle actin (Cat. # MXA95) and rabbit skeletal muscle actin (Cat. # AKL99) to 0.2 mg/ml with G-buffer (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0. 5 mM DTT)
2. Incubate both MXA95 and AKL99 actin on ice for 2 hours
   longer incubation is necessary for AKL99 to disperse nucleation bodies
3. Centrifuge the MXA95 and AKL99 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MXA95 and AKL99 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MXA95 and AKL99 actin samples to 0.1 mg/ml with G-buffer
7. Aliquot 20 µl (2 µg 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 4 conditions as shown in Table 1
8. Before beginning subtilisin treatment boil a beaker of water. The water should be boiling when the subtilisin reaction is complete to inactivate the protease

5. 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 CaCl2 to make a 31.25 µg/ml stock. Aliquot and store at -70ºC.
6. Dilute a tube of subtilisin stock to 2 µg/ml with G-buffer.
   (*Subtilisin analysis: 1:200 w/w subtilisin to actin = 10 ng subtilisin / 2ug actin*)

10. Add 10 ng of subtilisin to treated condition for all 4 samples
   (*Subtilisin should be diluted and added to sample <u>in less than 10 min</u>*)
11. Treat actin samples with subtilisin for 15 min
12. During treatment make PMSF (10 mM: 1.74 mg PMSF per ml of isopropanol)
13. After 15 min of subtilisin treatment, stop the reactions by adding 1 µl of PMSF to each sample (1-4)
14. Add 5 µl of 5x reducing sample buffer to each sample (1-4)
   NOTE: Immediately boil samples once reducing buffer is added, as dena turing the actin sample allows trace activity of subtilisin A to rapidly cleave actin.
15. Immediately place samples into boiling water. Leave samples for 5 min
16. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes

Table 1: Composition of Experimental Samples for Subtilisin Digestion

Sample	Name	Add Subtilisin	Add PMSF
1	ALK99	No	Yes
2	AKL99	Yes	Yes
3	MXA95	No	Yes
4	MXA95	Yes	Yes

17. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
18. Visualize with Coomassie staining. See Figure 3

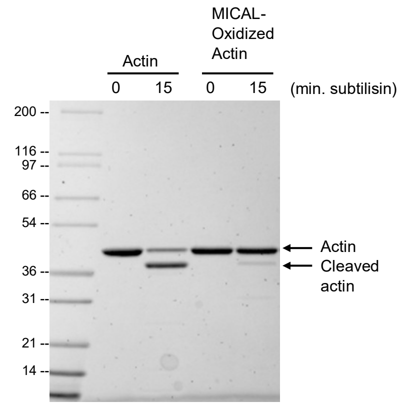

Figure 3. Subtilisin Assay on MICAL-Oxidized Actin vs Native Actin. Actin (Cat. # AKL99) and MICAL-oxidized actin (Cat. # MXA95) was diluted to 0.1 mg/ml (2.3 µM). 2 µg of each sample was then left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

Evaluating Methionine Oxidation of Actin: Sedimentation Application

Rationale:

When actin is oxidized at M44 its ability to polymerize is significantly diminished [3]. However, with increasing concentrations, oxidized actin can form augmented polymers [9]. Sedimentation assays can be used to measure the polymerization capabilities of native actin versus MICAL-oxidized actin.

Expected Results:

Purified MICAL-oxidized actin will polymerize less efficiently relative to native actin in an actin concentration dependent manner as shown in Table 2

Table 2: Expected % actin polymer formed with native versus oxidized actin

	% polymer unoxidized actin	% polymer MIC01 oxidized actin
0.1 mg/ml actin	80-90%	40-50%
0.4 mg/ml actin	85-95%	68-78%

Reagents Needed:

MICAL-oxidized Rabbit skeletal muscle actin (Cat. # MXA95): 1 mg is sufficient for 45 sedimentation reactions when used at 0.1 mg/ml and 11 reactions when used at 0.4 mg/ml
Rabbit skeletal muscle actin (Cat. # AKL99)
G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)
Precision red advanced protein assay (Cat. # ADV02)
2x polymerization buffer (5 mM Tris pH 7.5, 4 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)

Method:

1. Resuspend MICAL-oxidized rabbit skeletal muscle actin (Cat. # MXA95) and rabbit skeletal muscle actin (Cat. # AKL99) to 1.0 mg/ml with G-buffer (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0. 5 mM DTT)
2. Incubate both MXA95 and AKL99 actin on ice for 1 h
3. Centrifuge the MXA95 and AKL99 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MXA95 and AKL99 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MXA95 and AKL99 actin samples to the following conc. 0.8 mg/ml and 0.2 mg/ml with G-buffer
6. Prepare 2x polymerization buffer
7. Prepare four microcentrifuge tubes labeled 1-4
8. Prepare the following samples in the microcentrifuge tubes as shown in Table 3
9. Transfer 200 µl of each sample to tubes compatible for ultracentrifugation.
   Accurate loading is important for tube balance
10. Incubate at room temperature for 1 h
11. Centrifuge at 100,000 g in an ultracentrifuge for 1.5 h
12. After centrifugation remove the top 180 µl of supernatant from each sample and place into new 1.5 ml tubes labeled S1-S4
    Leave the remaining supernatant, as the pellet may become detached if disturbed

Table 3: Composition of Experimental Samples

sample	name	0.2mg/ml actin	0.8 mg/ml actin	2x pol buffer
1	AKL99 - 0.1 mg/ml	110 µl AKL99	0	110 µl
2	MXA95 - 0.1 mg/ml	110 µl MXA95	0	110 µl
3	AKL99 - 0.4 mg/ml	0	110 µl AKL99	110 µl
4	MXA95 - 0.4 mg/ml	0	110 µl MXA95	110 µl

13. Add 36 µl of 5x reducing sample buffer to tubes S1-S4
14. Resuspend pellets in 200 µl of 1x reducing sample buffer
15. Boil supernatant and pellet samples for 5 min
16. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes
17. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
    Use 40 µl of supernatant and pellet for samples 1 and 2, and 10 µl of each for samples 3 and 4 which will result in equal actin concentration loading between the 0.1 mg/ml and 0.4 mg/ml samples
18. Visualize with Coomassie staining. See Figure 4

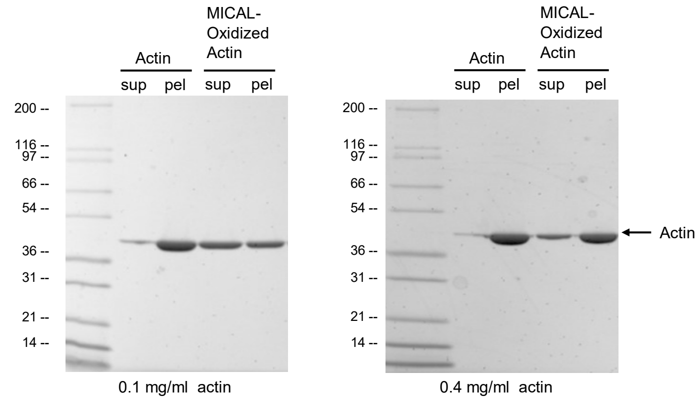

Figure 4. Actin Sedimentation Oxidized Versus Native Actin. Actin (Cat. # AKL99) and MIcal-oxidized actin was diluted to 0.2 mg/ml (4.6 µM) or 0.8 mg/ml (18.4 µM) (see method). Samples were then incubated with 2x polymerization buffer at room temperature. Samples were spun in an ultracentrifuge at 100,000 g for 1.5 h. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

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