V. 1.9

Pyrene Labeled Actin Protein (MICAL-Oxidized): Rabbit Skeletal Muscle

(Rabbit Muscle, > 95% Pure)
Cat. # MPAX1
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:	Polymerization assay Sedimentation assay

Online Datasheet Contains
MPAX1 (V1.9) and MPAX1-XL (V1.9)

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].

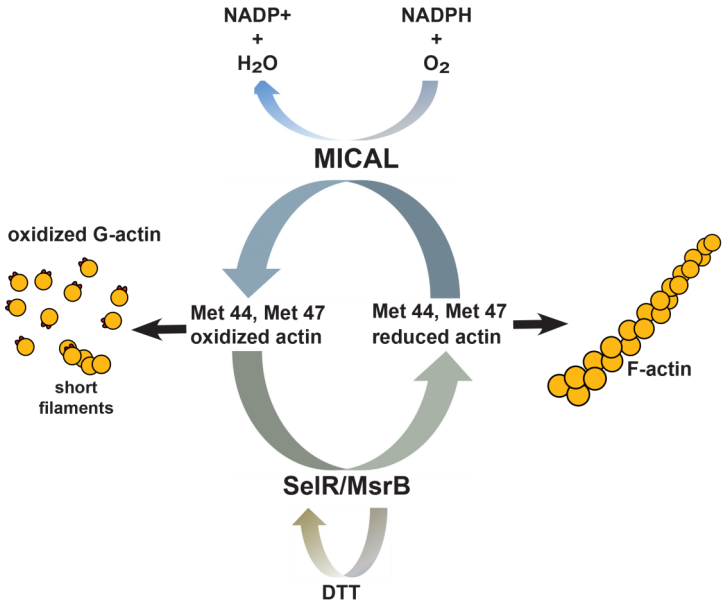

Figure 1. Actin Met 44 and Met 47 physiological redox system. A schematic diagram of the enzymes that control the physiological and reversible oxidation and reduction of methionines 44 and 47 of actin. Specifically, the MICAL family of proteins regulate oxidation of actin at Met 44 and Met 47. Conversely,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

Material

Purified rabbit muscle actin (Cat. # AP05) has been modified to contain covalently linked pyrene at the cysteine 374 residue. An N -(1-pyrene) iodoacetamide is used to label the actin protein. Pyrene labeling stoichiometry has been determined to be between 0.4-0.6 dyes per actin monomer.

Pyrene labeled rabbit skeletal muscle actin protein (MICAL-oxidized) (Cat. # MPAX1) has been enzymatically oxidized at methionines 44 and 47 (β-actin nomenclature) with the MICAL flavoprotein monoxygenase protein (Cat. # MIC01). MICAL-oxidized (pyrene labeled) 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 per tube; 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 (pyrene labeled) actin is a labile protein and should be handled with care. Diluted pyrene actin is stable for a maximum of 4 h at 4°C and should not be frozen. Avoid repeated freeze-thaw cycles and do not freeze below 10 mg/ml.

When stored and reconstituted as described, MPAX1 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**).

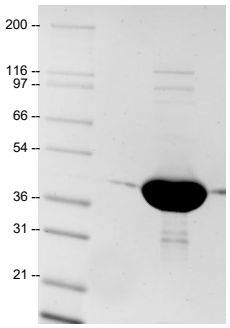

Figure 2. Pyrene labeled Actin Protein (MICAL-Oxidized) Purity Determination. A 50 µg sample of pyrene labeled actin protein (MICAL-oxidized) was separated by electrophoresis in a 4- 20% tris-glycine gel and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein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

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

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 (pyrene labeled) actin has reduced susceptibility to subtilisin A cleavage at M47/G48 by > 90% under the conditions described below.

Reagents Needed:
MICAL-oxidized (pyrene labeled) rabbit skeletal muscle actin (Cat. # MPAX1): 250 µg is sufficient for roughly 113 subtilisin reactions when used at 2 µg / rxn
Pyrene labeled rabbit skeletal muscle actin (Cat. # AP05)
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 (pyrene labeled) rabbit skeletal muscle actin (Cat. # MPAX1) and pyrene labeled rabbit skeletal muscle actin (Cat. # AP05) 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 MPAX1 and AP05 actin on ice for 2 hours
   longer incubation is necessary for AP05 to disperse nucleation bodies
3. Centrifuge the MPAX1 and AP05 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MPAX1 and AP05 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MPAX1 and AP05 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 denaturing 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
17. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel

Table 1: Composition of Experimental Samples for Subtilisin Digestion

Sample	Name	Add Subtilisin	Add PMSF
1	AP05	No	Yes
2	AP05	Yes	Yes
3	MPAX1	No	Yes
4	MPAX1	Yes	Yes

18. Visualize with Coomassie staining. See Figure 3

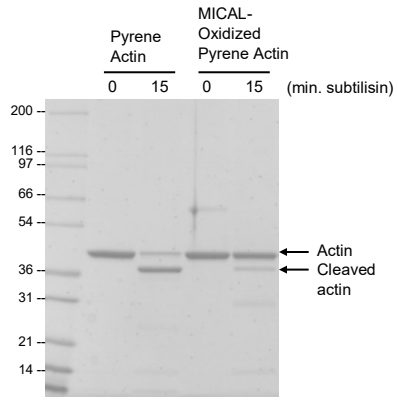

Figure 3. Subtilisin Assay on MICAL-Oxidized Pyrene Actin vs Native Pyrene Actin. Pyrene labeled actin (Cat. # AP05) and MICAL-oxidized (pyrene labeled) actin (Cat. # MPAX1) was diluted to 0.1 mg/ml (2.3 µM). 2 µg of each sample was them left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

Evaluating Methionine Oxidation of Actin: Polymerization 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]. Polymerization assays can be used to measure the polymerization capabilities of native actin versus MICAL-oxidized actin.

Expected Results:

Purified MICAL-oxidized (pyrene labeled) actin will polymerize less efficiently relative to native pyrene labeled 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
0.05 mg/ml actin	100%	<10 % of AP05
0.1 mg/ml actin	100%	40-55% of AP05

Reagents Needed:

MICAL-oxidized (pyrene-labeled) rabbit skeletal muscle actin (Cat. # MPAX1): 250 µg is sufficient for roughly 22 polymerization reactions when used at 0.05 mg/ml and 11 reactions when used at 0.1 mg/ml final.

Pyrene labeled rabbit skeletal muscle actin (Cat. # AP05)

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)

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. Resuspend MICAL-oxidized (pyrene labeled) rabbit skeletal muscle actin (Cat. # MPAX1) and pyrene labeled rabbit skeletal muscle actin (Cat. # AP05) 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 MPAX1 and AP05 actin on ice for 1 h
3. Centrifuge the MPAX1 and AP05 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MPAX1 and AP05 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MPAX1 and AP05 actin samples to the following conc. 0.1 mg/ml and 0.2 mg/ml with G-buffer
7. Prepare 2x polymerization buffer
8. Pipet 100 µl of G-buffer into two wells (A1-2) (control samples)
9. Pipet 100 µl of 0.1 mg/ml AP05 into two wells (A3-4) of a black 96 well plate
10. Pipet 100 µl of 0.1 mg/ml MPAX1 into two wells (B3-4) of a black 96 well plate
11. Pipet 100 µl of 0.2 mg/ml AP05 into two wells (A5-6) of a black 96 well plate
12. Pipet 100 µl of 0.2 mg/ml MPAX1 into two wells (B5-6) of a black 96 well plate
13. Place the 96 well plate into the fluorescent spectrophotometer and read the samples for 3 min to establish a baseline fluorescent measurement
14. After 3 min add 100 µl of 2x polymerization buffer to all wells with samples or blanks. (A1-6, B3-6)
15. Return the plate to the spectrophotometer and read the fluorescence every 30 s for 2 h

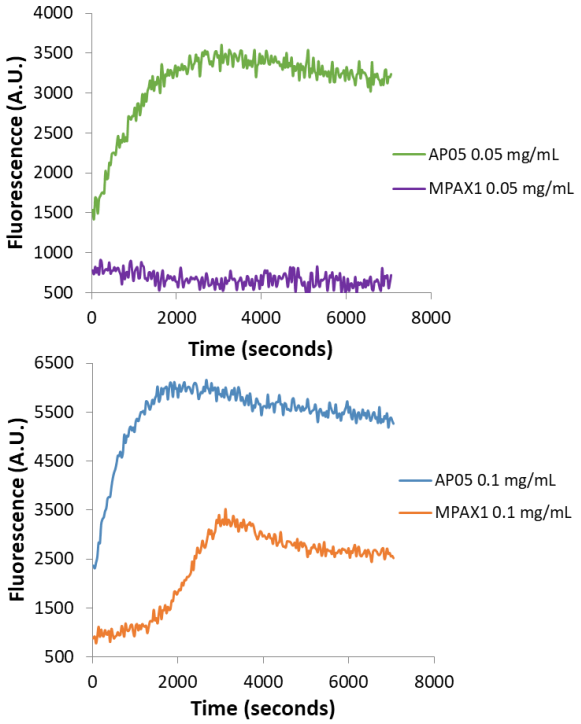

Figure 4. Actin Polymerization of Oxidized Pyrene Actin Versus Native Pyrene Actin. Pyrene-labeled actin (Cat # AP05) and MICAL-oxidized (pyrene labeled) actin (Cat. # MPAX1) were diluted to 0.1 mg/ml (2.3 µM) or 0.2 mg/ml (see method). Samples were then incubated with an equal volume of 2x polymerization buffer. Upon actin polymerization fluorescence was detected with a spectrophotometer. A.U. = arbitrary units

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.
3. 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.
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.
6. Grintsevich EE, Ge P, Sawaya MR, Yesilyurt HG, Terman JR, Zhou ZH, et al. Catastrophic disassembly of actin filaments via MICAL-mediated oxidation. *Nat Commun*. 2017;8(1):2183, 10.1038/s41467-017-02357-8.
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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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V. 1.9

Pyrene Labeled Actin Protein (MICAL-Oxidized): Rabbit Skeletal Muscle

(Rabbit Muscle, > 95% Pure)
Cat. # MPAX1-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:	Polymerization assay 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].

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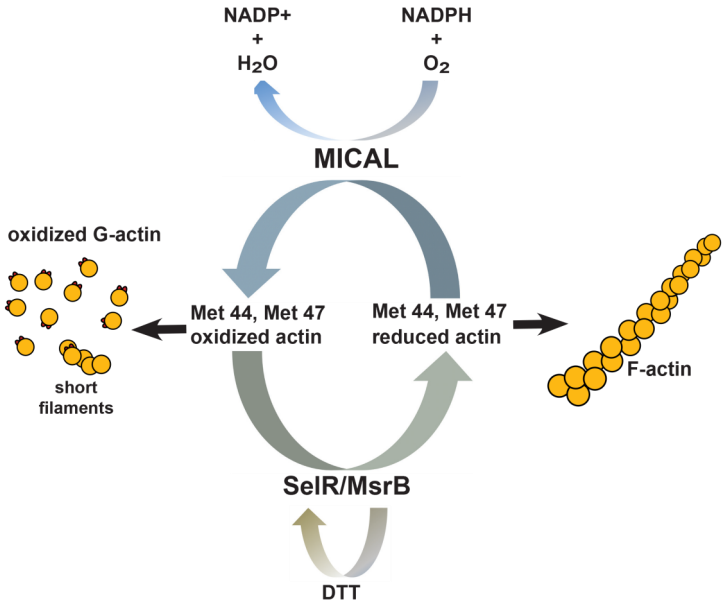

Figure 1. Actin Met 44 and Met 47 physiological redox system. A schematic diagram of the enzymes that control the physiological and reversible oxidation and reduction of methionines 44 and 47 of actin. Specifically, the MICAL family of proteins regulate oxidation of actin at Met 44 and Met 47. Conversely,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

Material

Purified rabbit muscle actin (Cat. # AP05) has been modified to contain covalently linked pyrene at the cysteine 374 residue. An N -(1-pyrene) iodoacetamide is used to label the actin protein. Pyrene labeling stoichiometry has been determined to be between 0.4-0.6 dyes per actin monomer.

Pyrene labeled rabbit skeletal muscle actin protein (MICAL-oxidized) (Cat. # MPAX1) has been enzymatically oxidized at methionines 44 and 47 (β-actin nomenclature) with the MICAL flavoprotein monoxygenase protein (Cat. # MIC01). MICAL-oxidized (pyrene labeled) 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 (pyrene labeled) actin is a labile protein and should be handled with care. Diluted pyrene actin is stable for a maximum of 4 h at 4°C and should not be frozen. Avoid repeated freeze-thaw cycles and do not freeze below 10 mg/ml.

When stored and reconstituted as described, MPAX1 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**).

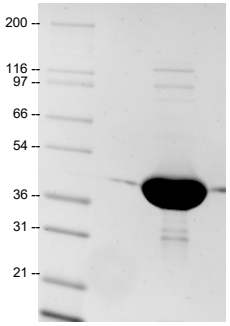

Figure 2. Pyrene labeled Actin Protein (MICAL-Oxidized) Purity Determination. A 50 µg sample of pyrene labeled actin protein (MICAL-oxidized) was separated by electrophoresis in a 4- 20% tris-glycine gel and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein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

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

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 (pyrene labeled) actin has reduced susceptibility to subtilisin A cleavage at M47/G48 by > 90% under the conditions described below.

Reagents Needed:
MICAL-oxidized (pyrene labeled) rabbit skeletal muscle actin (Cat. # MPAX1-XL): 1 mg is sufficient for roughly 454 subtilisin reactions when used at 2 μg / rxn
Pyrene labeled rabbit skeletal muscle actin (Cat. # AP05)
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 (pyrene labeled) rabbit skeletal muscle actin (Cat. # MPAX1) and pyrene labeled rabbit skeletal muscle actin (Cat. # AP05) 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 MPAX1 and AP05 actin on ice for 2 hours
   longer incubation is necessary for AP05 to disperse nucleation bodies
3. Centrifuge the MPAX1 and AP05 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MPAX1 and AP05 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MPAX1 and AP05 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 denaturing 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
17. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel

Table 1: Composition of Experimental Samples for Subtilisin Digestion

Sample	Name	Add Subtilisin	Add PMSF
1	AP05	No	Yes
2	AP05	Yes	Yes
3	MPAX1	No	Yes
4	MPAX1	Yes	Yes

18. Visualize with Coomassie staining. See Figure 3

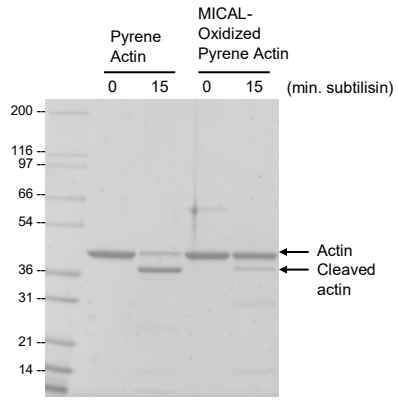

Figure 3. Subtilisin Assay on MICAL-Oxidized Pyrene Actin vs Native Pyrene Actin. Pyrene labeled actin (Cat. # AP05) and MICAL-oxidized (pyrene labeled) actin (Cat. # MPAX1) was diluted to 0.1 mg/ml (2.3 μM). 2 μg of each sample was them left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

Evaluating Methionine Oxidation of Actin: Polymerization 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]. Polymerization assays can be used to measure the polymerization capabilities of native actin versus MICAL-oxidized actin.

Expected Results:

Purified MICAL-oxidized (pyrene labeled) actin will polymerize less efficiently relative to native pyrene labeled 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
0.05 mg/ml actin	100%	<10 % of AP05
0.1 mg/ml actin	100%	40-55% of AP05

Reagents Needed:

MICAL-oxidized (pyrene-labeled) rabbit skeletal muscle actin (Cat. # MPAX1-XL): 1 mg is sufficient for roughly 90 polymerization reactions when used at 0.05 mg/ml and 45 reactions when used at 0.1 mg/ml final.

Pyrene labeled rabbit skeletal muscle actin (Cat. # AP05)

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)

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. Resuspend MICAL-oxidized (pyrene labeled) rabbit skeletal muscle actin (Cat. # MPAX1) and pyrene labeled rabbit skeletal muscle actin (Cat. # AP05) 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 MPAX1 and AP05 actin on ice for 1 h
3. Centrifuge the MPAX1 and AP05 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MPAX1 and AP05 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MPAX1 and AP05 actin samples to the following conc. 0.1 mg/ml and 0.2 mg/ml with G-buffer
7. Prepare 2x polymerization buffer
8. Pipet 100 µl of G-buffer into two wells (A1-2) (control samples)
9. Pipet 100 µl of 0.1 mg/ml AP05 into two wells (A3-4) of a black 96 well plate
10. Pipet 100 µl of 0.1 mg/ml MPAX1 into two wells (B3-4) of a black 96 well plate
11. Pipet 100 µl of 0.2 mg/ml AP05 into two wells (A5-6) of a black 96 well plate
12. Pipet 100 µl of 0.2 mg/ml MPAX1 into two wells (B5-6) of a black 96 well plate
13. Place the 96 well plate into the fluorescent spectrophotometer and read the samples for 3 min to establish a baseline fluorescent measurement
14. After 3 min add 100 µl of 2x polymerization buffer to all wells with samples or blanks. (A1-6, B3-6)
15. Return the plate to the spectrophotometer and read the fluorescence every 30 s for 2 h

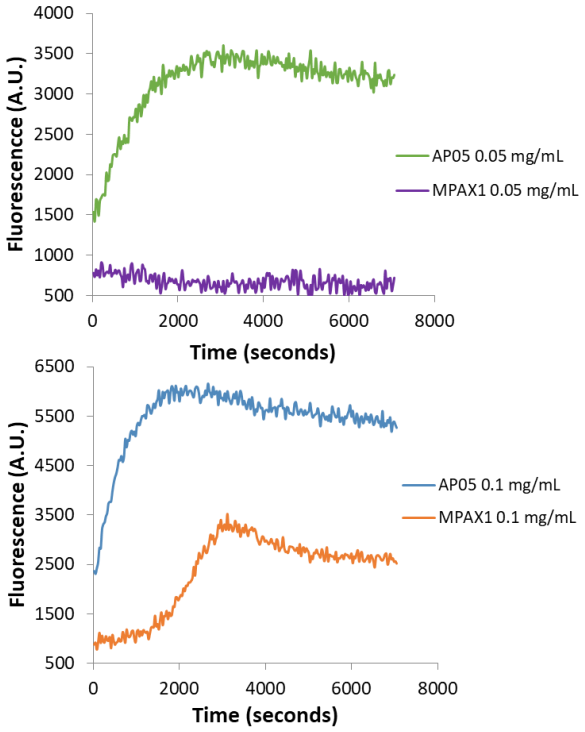

Figure 4. Actin Polymerization of Oxidized Pyrene Actin Versus Native Pyrene Actin. Pyrene-labeled actin (Cat # AP05) and MICAL-oxidized (pyrene labeled) actin (Cat. # MPAX1) were diluted to 0.1 mg/ml (2.3 µM) or 0.2 mg/ml (see method). Samples were then incubated with an equal volume of 2x polymerization buffer. Upon actin polymerization fluorescence was detected with a spectrophotometer. A.U. = arbitrary units

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