TC-FlAsH™ Expression Analysis Detection Kits

Catalog nos. A10067, A10068

Table 1. Contents and Storage information.

Material	Amount	Concentration	Storage	Stability
FlAsH Loading Buffer, Component A	1 mL	2X	• ≤–20ºC • Desiccate • Protect from light	When stored as directed the product is stable for at least 6 months.
Orange Total Protein Stain (A10067) or Red Total Protein Stain (A10068), Component B	1 vial	--		
Dimethylsulfoxide (DMSO), Component C	400 µL	--		
BenchMark™ Protein Ladder, Component D	90 µL	3X		
Number of reactions: Sufficient material is supplied for ten 17-well mini gels, based on the standard 12 µL reaction volume protocol below.				
Approximate fluorescence excitation and emission maxima: FlAsH dye 505 nm/530 nm when bound to TC-tagged protein, and 585 nm/620 nm (orange kit) or 650 nm/660 nm (red kit) for total protein labeling.				

Introduction

Expression analysis (EA) using FlAsH (Fluorescein Arsenical Hairpin) is a technique to identify tetracysteine (TC) tagged proteins and total proteins by SDS-PAGE. Protein samples are pre-labeled before electrophoresis by a combination of FlAsH, the biarsenical labeling technology first described by Tsien and coworkers,[1] and a total protein labeling dye. The combination of a site-specific labeling reagent and a total protein labeling reagent yields both western blot and Coomassie-like results on the same gel at the same time. The TC-tagged protein fluoresces green and is differentiated from total proteins that are labeled by an orange or red fluorescent dye. The choice of orange or red dye for total protein detection offers greater flexibility in matching the TC-FlAsH™ Expression Analysis Detection Kits to a gel imaging device.

The EA-FlAsH technique involves the same basic sample preparation as normal SDS-PAGE; however no post-electrophoretic gel staining is required. The FlAsH Loading Buffer (Component A) contains typical SDS-PAGE loading buffer ingredients such as reducing agents and also

includes the TC-Tag binding dye, FlAsH. The Total Protein Stain (Component B) contains a total protein staining dye that eliminates the need to perform gel post-staining allowing gel imaging directly through the cassette immediately following electrophoresis. Removing the need for gel staining saves time and reagents while still maintaining excellent sensitivity of protein detection. The TC-FlAsH™ Expression Analysis Detection Kits allow detection of single nanogram quantities of protein per band. Furthermore, imaging the gel directly through the cassette eliminates tearing or ripping of the gel. Even if the gel is removed from the cassette for imaging, gel handling is greatly minimized.

The TC-FlAsH™ Expression Analysis Detection Kits provide a rapid alternative to western blotting and total protein gel staining. In just minutes cell pellets, crude lysates, or purified proteins are labeled and ready for SDS-PAGE analysis using NuPAGE® Novex® Bis-Tris, Novex® Tris-Glycine, or E-PAGE™ gels. Following electrophoresis, the gel is ready to image without any processing or post-gel fixation. In the FlAsH channel (488 nm/520 nm or mid-UV), only TC-tagged proteins are detected, whereas in the orange (532 nm/580 nm or mid-UV) or red (633 nm/675 nm) channel, the total protein profile of the sample is observed. The TC-FlAsH™ product enables expression analysis of a TC-tagged protein of interest from mammalian or bacterial cells in about the same time it takes to run a standard SDS-PAGE gel.

Before You Begin

Materials Required but Not Provided

- Bacterial or mammalian cells expressing the TC-tagged protein or purified protein containing the TC-Tag
- 1% SDS (for bacterial cells) or phosphate buffer saline (PBS, for mammalian cells)
- Water bath or heat block set at 70ºC
- SDS-PAGE mini gel (NuPAGE® Novex®, Tris-Glycine, or E-PAGE™ gels)
- Fluorescent imager or UV transilluminator with appropriate filter sets

Reagents

1.1 Allow vials to warm to room temperature before opening. Vortex to mix thoroughly.

1.2 Add 350 µL DMSO (Component C) to the vial containing Total Protein Stain (Component B) to prepare 6X Total Protein Stain. Vortex to thoroughly resuspend.

Caution

Exercise caution when handling the product as it contains trace amounts of arsenic. Wear protective clothing, eyewear, and gloves suitable for use with dimethylsulfoxide (*e.g.* nitrile gloves) when handling the FlAsH Loading Buffer.

Treat accidental spills of the FlAsH Loading Buffer on surfaces with 10% bleach for 10 minutes and then carefully clean up. Discard arsenic-containing waste according to your institution's guidelines.

Treat accidental contact of the FlAsH Loading Buffer with human skin by washing excess reagent with soap and water as soon as possible. Consult a physician following contact. Do not treat arsenic skin exposure with EDT (1,2-ethanedithiol) as this may promote uptake of the reagent into the body.

Disposal

Discard all excess reagents that contain or have come in contact with arsenic compounds according to your institution's guidelines and all applicable local, state, and federal requirements.

In general, we recommend disposing of protein samples labeled with the TC-FlAsH Reagent and polyacrylamide gels containing protein samples labeled with the FlAsH Reagent as hazardous waste. For specific disposal requirements in your area, consult your safety officer.

Experimental Protocol

When fused to a gene of interest, the TC (tetracysteine) tag allows specific recognition of the expressed fusion protein by the TC-FlAsH™ detection reagent. The TC-FlAsH™ reagent binds to the tetracysteine motif, which is rarely seen in naturally occurring proteins, allowing specific fluorescent labeling of recombinant proteins fused to the TC-Tag (Figure 1). In the TC-FlAsH™ system, the tetracysteine motif is Cys-Cys-Pro-Gly-Cys-Cys as this motif has been shown to have a higher affinity for and more rapid binding to biarsenical compounds as well as enhanced stability compared to other characterized motifs.[2]

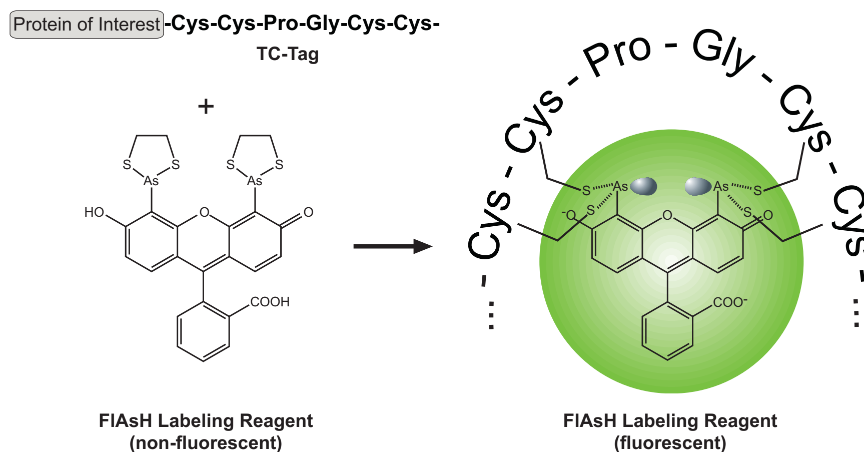


Figure 1. Interaction between the TC-Tag and FlAsH reagent.

Sensitivity

The TC-tagged protein should be expressed at >25 ng/µL to obtain good detection. The protein detection sensitivity for each fluorescent dye is listed in Table 2.

Table 2. Detection sensitivity chart.

Fluorescent Dye	Wavelength	Protein
TC-FlAsH™ (in the cassette)	488 nm	4 ng*
	UV	60 ng*
TC-FlAsH™ (out of the cassette)	488 nm	250 pg*
	UV	1 ng*
Total protein (at 532 or 633 nm)	Orange IN	100 ng**
	Orange OUT	12 ng**
	Red IN	100 ng**
	Red OUT	50 ng**

*Indicates amount of TC-tagged protein
**Indicates total amount of protein loaded

Adding the TC-Tag to Construct

You can add the TC-Tag at the 5′ or 3′ or at an internal site of your gene of interest (N-or C-terminus, or at an internal site of your protein of interest) using the following methods:

- Gateway® Technology

  Gateway® is a universal cloning technology that takes advantage of the site-specific recombination properties of bacteriophage lambda[3] to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest using the Gateway® Technology, simply:

  1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone. Many entry vectors are available from Invitrogen to facilitate generation of entry clones.
  2. Generate an expression clone by performing an LR recombination reaction between the entry clone and a destination vector of choice containing the TC-Tag. Destination vectors for use with the TC-FlAsH™ system are available from Invitrogen.

  For more information about Gateway® Technology and performing the LR recombination reaction, refer to the Gateway® Technology manual available from www.invitrogen.com or by contacting Technical Support.

- Directional TOPO® Cloning

  The Champion™ pET Directional TOPO® Expression Kits from Invitrogen utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for high-level, T7-regulated expression of TC-tagged proteins in *E. coli*.

  For more information about Directional TOPO® Cloning, refer to the Champion™ pET Directional TOPO® Expression Kit manual available from www.invitrogen.com or by contacting Technical Support.

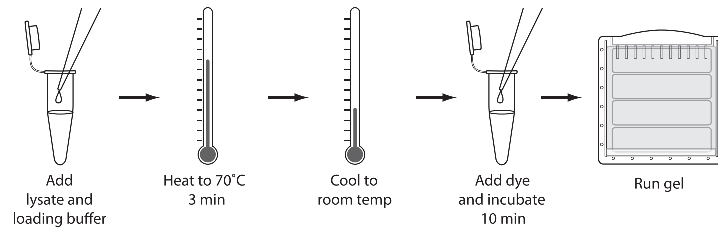
- PCR

  Design PCR primers to clone your gene of interest in frame with the TC-Tag. Example of the TC-Tag sequence is shown below. Produce your PCR product and clone your PCR product into a vector of choice.

  5′-TGCTGCCCTGGCTGCTGC-3′

Note You may prepare samples and perform the labeling reaction using the Standard protocol (**Preparing Samples** followed by **Setting Up the Labeling Reaction**) or **One-tube Protocol** (Figure 2) depending on your experimental needs. Sufficient reagents are provided to run ten 17-well gels, using the Standard protocol for 12 µL reactions. Using the One-tube protocol reduces this amount by half. The One-tube protocol allows you to quickly evaluate the TC-tagged protein expression level using a small aliquot of the culture. Once the desired expression level is achieved, use the Standard Protocol to prepare samples for detailed analysis.

Standard protocol:



"One-tube" protocol:

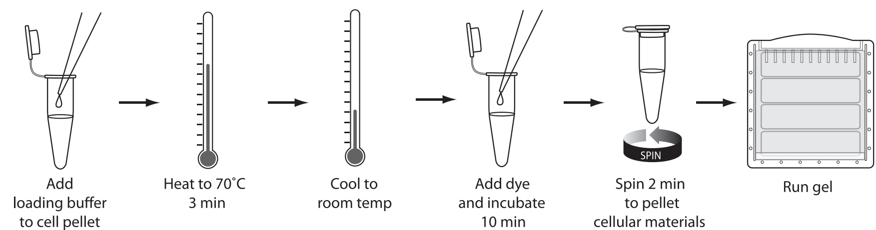


Figure 2. Experimental outline for the Standard and One-tube protocol.

Preparing Samples

2.1 Harvest cells expressing the TC-tagged protein of interest and pellet cells using centrifugation.

2.2 Remove supernatant/media from the cell pellet.

Note: BSA binds to the FlAsH dye. Wash cells well to remove any BSA.

2.3 Add 1% SDS or 1X PBS to resuspend/lyse your cells (some proteins may not be soluble in some buffers, use an appropriate buffer).

2.4 If cells are resuspended in 1X PBS, sonicate the cells on ice for 4 × 10 second bursts at 50% maximum amplitude to lyse.

If cells are resuspended in 1% SDS, vortex to completely lyse cells. Heat the lysate at 70ºC for 5 minutes, vortex again, and repeat until cells are lysed.

2.5 Centrifuge the lysates at >13,000 × g for 5 minutes to pellet cellular debris.

2.6 Transfer the supernatant to a clean tube and use the supernatant as a 3X protein sample for reaction volume calculations. Proceed to the labeling reaction.

Setting Up the Labeling Reaction

Instructions to set up a standard **12 µL labeling reaction** are described below. Use the BenchMark™ Protein Ladder (Component D) as a positive control (4 µL in a standard 12 µL labeling reaction) for total protein detection.

Note: To reduce image saturation, the BenchMark™ Protein Ladder included with this kit is supplied at half the concentration as compared to the BenchMark™ Protein Ladder available separately (Invitrogen Cat. no. 10747-012).

3.1 Heat the FlAsH Loading buffer (Component A) to 70ºC (<1 minute) and vortex briefly.

3.2 Add the following components to 0.7 mL microcentrifuge tubes:

Reagents	**Sample**	**Positive Control**
Protein lysates (3X, step 2.6)	4 µL	--
BenchMark™ Protein Ladder (Component D)	--	4 µL
Warm FlAsH Loading Buffer (Component A)	6 µL	6 µL
Heat samples at 70ºC for 3 minutes.		
Centrifuge briefly to collect the sample and cool to room temperature.		
6X Total Protein Stain (Component B, step 1.2)	2 µL	2 µL
Total Volume:	12 µL	12 µL

3.3 Mix by brief vortexing or pipetting up and down. Centrifuge briefly to collect the sample.

3.4 Incubate at room temperature for 10 minutes, away from direct sunlight.

3.5 Load samples and the BenchMark™ Protein Ladder (12 µL/lane) onto an appropriate SDS-PAGE gel, and perform electrophoresis as recommended by the manufacturer.

Note: To visualize a protein ladder in the FlAsH channel, load 4-5 µL/lane (for a 10- or 15-well mini gel) of BenchMark™ Fluorescent Protein Ladder (Invitrogen Cat. no. LC5928, see Figure 3).

3.6 After electrophoresis, proceed immediately to **Imaging and Analysis**.

One-tube Protocol

4.1 Heat the FlAsH Loading buffer (Component A) to 70ºC (<1 minute) and vortex briefly.

4.2 Remove 50 µL cells from your culture expressing the TC-tagged protein.

Note: BSA binds to the FlAsH dye. Wash cells well to remove any BSA.

4.3 Centrifuge the cells at 8,000 × g for 2 minutes. Discard the supernatant.

4.4 Resuspend the cell pellet in 8 µL 1X PBS or 1% SDS.

4.5 Add the following components to 0.7 mL microcentrifuge tubes:

Reagents	**Sample**	**Positive Control**
Cell pellet (step 4.4)	8 µL	--
BenchMark™ Protein Ladder (Component D)	--	8 µL
Warm FlAsH Loading Buffer (Component A)	12 µL	12 µL
Vortex to completely resuspend the pellet.		
Heat samples at 70ºC for 3 minutes.		
Centrifuge briefly to collect the sample and cool to room temperature.		
6X Total Protein Stain (Component B, step 1.2)	4 µL	4 µL
Total Volume:	24 µL	24 µL

4.6 Mix by vortexing briefly or pipetting up and down.

4.7 Incubate at room temperature for 10 minutes, away from direct sunlight.

4.8 Centrifuge at 13,000 × g for 2 minutes to pellet cellular debris.

4.9 Load the appropriate volume of supernatant onto a suitable SDS-PAGE gel and perform electrophoresis as recommended by the manufacturer.

Note: To visualize a protein ladder in the FlAsH channel, load 4-5 µL/lane (for a 10- or 15-well mini gel) of BenchMark™ Fluorescent Protein Ladder (Invitrogen Cat. no. LC5928, see Figure 3).

4.10 After electrophoresis, proceed immediately to **Imaging and Analysis**.

Imaging and Analysis

5.1 After electrophoresis is complete (as indicated by the dye band reaching the bottom of gel), remove gel cassette from the electrophoresis unit.

5.2 Rinse the gel cassette in deionized water. Wipe dry the cassette with a paper towel.

5.3 For TC-tagged protein detection, place the gel cassette on a fluorescent imager at appropriate excitation and emission (Table 3), or an UV transilluminator equipped with a standard camera and appropriate filters, and obtain the image.

You should see fluorescent bands of TC-tagged proteins and the gel cassette should have minimal background as shown in Figure 3.

Note: The sensitivity of detection is higher when the gel is visualized and imaged after removal from the cassette (Table 2).

5.4 For total protein expression detection with UV transilluminator, use the Orange kit as the Red kit is not UV compatible for total protein detection.

For total protein detection with a fluorescent imager, use the Orange or Red kit depending on the excitation and filter options of your imaging system.

Table 3. Imaging recommendations.

Detection	Excitation peak	Emission peak	Recommended Filters
TC-tagged protein	500 nm Mid-UV	535 nm	520 nm LP 500-600 nm BP Kodak #15
Orange total protein	580 nm Mid-UV	620 nm	580 nm LP 580-660 nm BP Kodak #22
Red total protein	650 nm	665 nm	675 nm LP

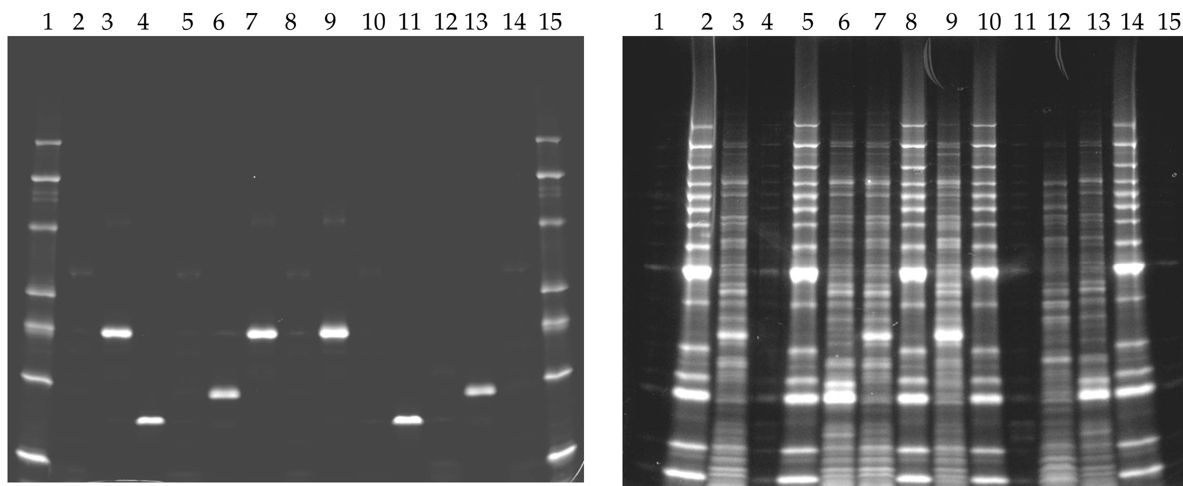


Figure 3. Purified TC-tagged proteins, crude lysates containing TC-tagged protein, and BenchMark™ Protein Ladder were subjected to protein detection using the TC-FlAsH™ Expression Analysis Detection Kit - Red (Cat. no. A10068). Samples were analyzed on a 4-20% Tris-Glycine SDS-PAGE gel and imaged using a Fuji FLA3000 laser scanner. With FlAsH detection (Panel A), only TC-tagged proteins appear with the Benchmark Fluorescent Protein Ladder while with total protein detection (Panel B), all proteins, including TC-tagged proteins are detected.
Panel A: TC-tagged proteins and Benchmark Fluorescent Protein Ladder visualized with 473 nm excitation with 520 nm long pass emission filter. **Panel B**: Total protein detection obtained with 633 nm excitation with 675 nm long pass emission filter.
Lanes 1, 15: BenchMark™ Fluorescent Protein Ladder (4 µL); Lanes 2, 5, 8 14: BenchMark™ Protein Ladder (200 ng/band); Lanes 3, 7: Lysate expressing TC-tagged CFP protein (4 µL); Lane 4: Purified TC-tagged ACP protein (200 ng); Lanes 6, 13: Lysate expressing TC-tagged calmodulin protein (4 µL); Lane 9: Lysate expressing TC-tagged GFP protein (4 µL); Lane 10: BenchMark™ Protein Ladder (100 ng/band); Lane 11: Purified TC-tagged ACP protein (100 ng); Lane 12: *E. coli* lysate (4 µL)

References

1. Science 281, 269 (1998); **2.** J. Am. Chem. Soc. 124, 6063 (2002); **3.** Ann. Rev. Biochem. 58, 913 (1989).

Product List **Current prices are available from www.invitrogen.com or from our Customer Service Department**

Catalog no.	Product Name	Unit Size
A10067	TC-FlAsH™ Expression Analysis Detection Kit - Orange *orange fluorescent in-gel detection of TC-tagged and total protein*……………………………………………......	1 kit
A10068	TC-FlAsH™ Expression Analysis Detection Kit - Red *red fluorescent in-gel detection of TC-tagged and total protein*……………………………………………......	1 kit
LC5928	BenchMark™ Fluorescent Protein Ladder…………………………………………….......	125 µL

A large variety of NuPAGE® Novex®, Tris-Glycine, and E-PAGE™ gels for SDS-PAGE analysis is available from Invitrogen. For details, visit www.invitrogen.com/Novex1D. For generating TC-tagged proteins using Gateway® entry and destination vectors, visit www.invitrogen.com/gateway.

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