

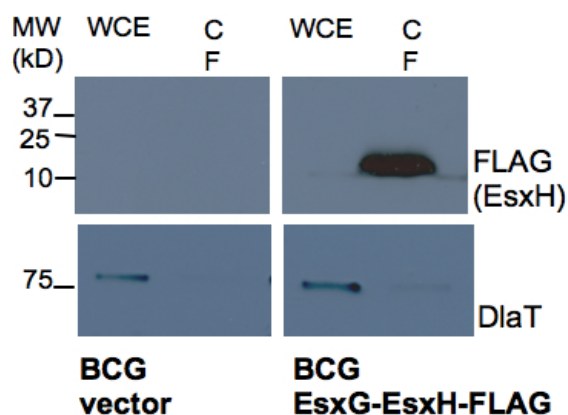
## Analysis of Mycobacterial Protein Secretion

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**[Abstract]** *Mycobacterium tuberculosis* (Mtb) is the causative agent of tuberculosis. Analysis of proteins secreted by Mtb has been of interest to the field of tuberculosis research since certain secreted proteins interact with the host to promote virulence, while others may be important antigens or serve as biomarkers of infection. Here, we describe a protocol to prepare whole cell extracts (WCE) and short term culture filtrate (CF) from Mtb or the vaccine strain *Mycobacterium bovis*- bacillus Calmette- Guérin (BCG) (Mehra *et al.*, 2013). These are both slow growing mycobacteria, but the same basic procedure can easily be adapted to analyze secreted proteins from rapidly growing mycobacteria, such as *Mycobacterium smegmatis* (Msmeg), a non-pathogenic species commonly used in the laboratory. The fractions obtained can be analyzed by western blotting to examine proteins of interest or by mass spectrometry if antibodies are not available or to examine the entire secretome. Genetic knockout mutants for the gene of interest serve as a negative control. Additionally, levels of a cytosolic protein such as the chaperone GroEL or the pyruvate dehydrogenase E2 component sucB (Rv2215/dlaT) should be assessed in the CF fraction to rule out the possibility that a positive signal in CF is due to bacterial lysis (see Figure 1). By varying the growth conditions of the strain, this *in vitro* secretion assay can be used to examine conditions that alter the secretome. We are thankful to Magnus Stiededal for helpful tips on TCA (trichloroacetic acid) precipitation.



**Figure 1. Western analysis of secretion of EsxH by BCG.** BCG containing an empty vector control and EsxG-EsxH-FLAG expression construct (FLAG tag at C terminal of EsxH) Copyright © 2014 The Authors; exclusive licensee Bio-protocol LLC.

were analyzed for presence EsxH by anti-FLAG western in WCE and CF prepared as described in the protocol. Dlat was used as a loading control to indicate the degree of bacterial lysis.

## **Materials and Reagents**

*Note: All work with live Mtb must be performed in a Biosafety Level 3 (BSL3) facility.*

1. Middlebrook 7H9 Broth (Difco, catalog number: 271310)
2. Tween-80 (Sigma-Aldrich, catalog number: P4780)
3. Glycerol (Sigma-Aldrich, catalog number: G5516)
4. Albumin-dextrose-catalase (ADC) (BD, catalog number: 212352)
5. Oleic-albumin-dextrose-catalase (OADC) (BD, catalog number: 212351)
6. Potassium phosphate (monobasic) ( $\text{KH}_2\text{PO}_4$ ) (Sigma-Aldrich, catalog number: P9791)
7. L-asparagine monohydrate (Sigma-Aldrich, catalog number: A8381)
8. Citric acid monohydrate (Sigma-Aldrich catalog number: C1909)
9. Ferric ammonium citrate (Sigma-Aldrich catalog number: F5879)
10. Zinc sulfate monohydrate ( $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ ) (Sigma-Aldrich, catalog number: 96495)
11. Magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) (Sigma-Aldrich, catalog number: 230391)
12. Chelex 100 resin (Bio-Rad Laboratories, catalog number: 142-2822)
13. 100x Halt Protease Inhibitor single use cocktail (Pierce, catalog number: 1860932)
14. 100% trichloroacetic acid (TCA) (pre-chilled prior to use) (Sigma-Aldrich, catalog number: 49010)
15. Acetone (pre-chilled prior to use) (Sigma-Aldrich, catalog number: 32201)
16. Phosphate buffered saline (PBS) (Life Technologies, Gibco®, catalog number: 10010-023)
17. Bromophenol Blue, sodium salt (US Biological, catalog number: 12370)
18. Tris (MP Biomedicals, catalog number: 02194855)
19. Sodium Dodecyl Sulfate (SDS) (US Biological, catalog number: 18220)
20. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E6758)
21.  $\beta$ -mercaptoethanol (2-ME) (Sigma-Aldrich, catalog number: M6250)
22. 7H9 complete media (see Recipes)
23. Sauton's media (see Recipes)
24. Chelated sauton's media (an alternative minimal media for mycobacterial growth) (see Recipes)
25. Protein extraction buffer (see Recipes)
26. 5x SDS-PAGE sample buffer (see Recipes)

## **Equipment**

1. Autoclave
2. Steriflip-GV filter units (0.22 µm pore size) (Millipore, catalog number: SE1M179M6)
3. 20 ml syringes (BD, catalog number: 302830)
4. 0.22 µm syringe filter units (33 mm) (Millipore, catalog number: SLGV033RS)
5. Disposable Sterile Filter system (1 L, 0.22 µm pore size) (Corning, catalog number: 09761104)
6. 0.1 mm zirconia/silica beads (Bio Spec Products, catalog number: 11079101z)
7. 30 ml square media bottles (Nalgene®, catalog number: NE/2019-0030)
8. 125 ml square media bottles (Nalgene®, catalog number: NE/2019-0125)
9. 50 ml falcon tubes (Corning, catalog number 430290)
10. 15 ml falcon tubes (Corning, catalog number: 430052) with plug seal caps  
*Note: These 50 ml tubes are compatible with organic solvents and high speed centrifugation. Falcon tubes with these features can be used from different vendors.*
11. Microtubes (2 ml screw cap with O rings) (SARSTEDT AG, catalog number: 72.693)
12. Spectrophotometer
13. Centrifuge with swinging bucket rotor for spinning down bacterial cultures (for example, Beckman Coulter, model: Allegra X-15R; bench top centrifuge with SX4750 rotor)  
*Notes:*
  - a. *Msmeg and BCG should be handled according to institutional standards of practice for biosafety.*
  - b. *Mtb cultures should be handled in biosafety level 3 facilities according to institutional standards of practice.*
  - c. *Centrifuging BCG and Mtb requires appropriate aerosol containment.*
14. Beckman Aerosolve® canisters to contain aerosols during centrifugation of mycobacterial cultures (e.g. Beckman Coulter, catalog number: BK359232)
15. 37 °C shaking incubator
16. Aerosol containment units for shaking BCG and Mtb liquid cultures in the shaking incubator  
*Note: Incubator should be placed in BSL3 facility for Mtb cultures.*
17. High speed centrifuge for 50 ml polypropylene falcons used for TCA precipitation of CF (e.g. Beckman Coulter centrifuge with JLA16.2 rotor)
18. 50 ml falcon adaptors for rotor JLA16.2
19. Bead beater (Bio Spec Products, model: Minibead beater 16;  
[http://www.biospec.com/product/34/mini\\_beadbeater/](http://www.biospec.com/product/34/mini_beadbeater/))
20. Standard table top centrifuge with refrigeration

21. Heating block for Eppendorf tubes (set to 95 °C)
22. pH meter

### **Acronyms**

1. Mtb: *Mycobacterium tuberculosis*
2. BCG: *Mycobacterium bovis* bacillus Calmatte- Guérin
3. Msmeg: *Mycobacterium smegmatis*
4. WCE: Whole cell extract
5. CF: Culture filtrate
6. DltA: Rv2215/pyruvate dehydrogenase E2 component sucB protein of mycobacteria
7. TCA: Trichloroacetic acid
8. BSC: Biosafety cabinet
9. OD<sub>600</sub>: Absorbance or Optical Density at wavelength of 600 nm

### **Procedure**

#### **A. Inoculation and growth of mycobacterial cultures**

1. Inoculate liquid cultures of mycobacteria from frozen stocks, prepared from mid-log phase mycobacterial cultures (OD<sub>600</sub> between 0.5 to 1.0) that were frozen in 18% glycerol at -80 °C.
  - a. For inoculation, the doubling rate of the mycobacterial species needs to be kept in mind. Msmeg doubles approximately every 3 h, whereas BCG and Mtb double approximately every 20 h in 7H9 complete media at 37 °C.
  - b. The cultures are inoculated in 10 ml of 7H9 complete media in 30 ml square media bottles. Include appropriate antibiotics if required e.g. antibiotics can be included at appropriate concentration for selecting a plasmid when growing a transformed mycobacterial strain.
  - c. The mycobacterial cultures are incubated at 37 °C with slow shaking at a speed of 90 rpm. BCG and Mtb cultures are placed in aerosol containment in a shaking incubator.
  - d. For Msmeg, inoculate 200 µl from glycerol stock and grow overnight to saturation. Dilute the over-night culture 1:40 into fresh 7H9 complete media and grow to mid-log phase. For BCG and Mtb, inoculate 0.5 ml from frozen stock. It will take several days (~4 days) for the culture to reach mid-log phase and the exact time depends upon the initial inoculum density and growth conditions.

2. Measure the OD<sub>600</sub> of the culture. Determine the culture volume required to give a final starting OD<sub>600</sub> of at least 0.25 when resuspended in 25 ml of Sauton's media [culture volume in ml = 25 ml x desired OD<sub>600</sub> (e.g. 0.25)/OD<sub>600</sub> of the experimental culture].
3. Transfer this volume of culture media to a 15 ml falcon tube and pellet the bacterial cells by centrifuging at 1500 x g for 7 min at room temperature. Remove the supernatant and resuspend the pellet in PBS. Again centrifuge at 1,500 x g for 7 min. This wash step is important to remove albumin carried over from 7H9 complete media.

*Notes:*

- a. As per our institutional standards of practice for centrifuging Mtb and BCG cultures, falcon tubes are placed in centrifuge adaptors that are put in Aerosolve® canisters (mentioned in Equipment, item 14) inside class II biosafety cabinet (BSC II).*
  - b. These are removed from the hood and loaded into the rotor and centrifuged.*
  - c. After spin, the whole Aerosolve® canister with the falcon tubes is carried back to the BSC II hood and opened.*
  - d. This procedure contains aerosols produced during centrifugation. Mtb cultures should be handled in BSL3 facility.*
4. Remove the supernatant and resuspend the bacterial pellet in 1 ml of Sauton's media. Transfer this to 125 ml square media bottle containing 24 ml of Sauton's media (include appropriate antibiotics in Sauton's media as required). Incubate in the shaking incubator at 37 °C.

*Notes:*

- a. Sauton's media is a minimal media in which mycobacteria grow more slowly than they do in 7H9 complete media.*
  - b. Alternatively, if the effect of metal ions on secretion is to be studied, one can use chelated Sauton's media as the minimal media and add defined concentrations of metal ions.*
5. Monitor OD<sub>600</sub> of the culture. Grow the culture to between 0.5 to 0.8 OD<sub>600</sub>. It takes about 6 h for Msmeg and 48 h for BCG and Mtb. This procedure could be modified to inoculate the bacteria at a lower density and allow them to grow for a longer period of time, depending upon the goals of the experiment.

## B. Harvesting the bacterial culture and preparation of the WCE

1. After incubating in Sauton's media as described above, determine the OD<sub>600</sub>.
2. Calculate the volume corresponding to 5 OD<sub>600</sub> units. For example, if the OD<sub>600</sub> of the culture is 0.5, then 10 ml will correspond to 5 OD units (desired volume = 5/OD<sub>600</sub> of the experimental culture).

3. Transfer 5 OD<sub>600</sub> of the culture into a 15 ml falcon tube (to be used to prepare the WCE). Transfer the remaining culture into a 50 ml falcon tube (to be used to precipitate proteins from the CF). Centrifuge both tubes at 2,000 x g for 5 min at 4 °C.
4. Pool the supernatant from both the 15 and 50 ml falcons into one unused 50 ml falcon tube and store on ice until you are ready to process it further (see below, preparation of CF in step C).
5. Wash the pellet in the 15 ml falcon with 5 ml of PBS by resuspending and centrifuging at 2,000 x g, 5 min, 4 °C.
6. Discard the supernatant and resuspend the pellet again in 1 ml of PBS and transfer it to a 2 ml Sarstedt tube with O ring in the screw cap (for containing aerosols during bead beating).
7. Spin on a table top centrifuge at 3,000 x g, 5min and discard the supernatant. To the pellet add 300 µl of protein extraction buffer with protease inhibitors. Resuspend the pellet by pipetting.
8. Dispense 100 µl of 0.1 mm zirconia/silica beads into a 1.7 ml eppendorf and add this to the Sarstedt tube containing the pellet.
9. Using a bead beater contained within a class II bio-safety cabinet (please read the bead beater manual for the operating procedure and also see the link provided in Equipment, item 19), bead beat the sample for 1 min at 3,450 oscillations/min and place it on ice for 2 min to cool. Repeat this twice. This sample is the WCE. Centrifuge the lysate at full speed for 2 min in a microcentrifuge to settle the froth before opening the tube.

*Notes:*

- a. *Alternate bead beaters may have different speeds and hence the user should optimize bead beating (speed and time) for maximal lysis of mycobacterial pellet.*
  - b. *The degree of lysis can be established by plating the bacteria before and after bead beating for colony forming units.*
10. For SDS-PAGE, add 60 µl of 5x SDS-PAGE sample buffer to the Sarstedt tube and then gently mix by inversion. Incubate at 95 °C for 5 min. The heating step helps to denature the sample and also sterilize mycobacterial lysate after bead beating. The mycobacterial WCE can be stored at -80 °C.
- Note: The WCE of Mtb can generally be transferred out of BSL3 facility at this step as long as that is consistent with the institutional standards of practice.*
11. Before use, thaw at room temperature and then heat at 95 °C for 5 min. 20 µl of the WCE is often sufficient for SDS-PAGE and western analysis, although this amount will depend upon the target protein levels and the sensitivity of the detection method.

### C. TCA precipitation of the CF

12. Filter the culture supernatant from step B9 through 0.22  $\mu$ M filters using a 25 ml syringe into a fresh 50 ml falcon tube.

#### Notes:

- a. *Alternatively, it can be filtered by vacuum using 50 ml Steriflip-GV filter units (0.22  $\mu$ M) to remove any bacteria from the supernatants.*
  - b. *This can be done twice depending on the turbidity of the supernatant.*
  - c. *Double filtration may also be required depending upon the standard operating procedures of the facility to transfer the CF from the BSL3 facility.*
18. Pre-chill trichloroacetic acid (TCA) on ice. Add TCA to a final concentration of 12% to the CF (3 ml of 100% TCA to the 25 ml sample). Invert the tube several times to mix and put the falcon tube at 4 °C overnight. Ensure the falcon tubes used for precipitation are compatible with organic solvents and high speed centrifugation.
  19. Spin the CF at 15,000 x g for 15 min at 4 °C.
  20. Discard the supernatant carefully to avoid losing the pellet.
  21. Wash the remaining TCA by adding 5 ml acetone (pre-chilled at 4 °C) slowly along the sides of the tube.
  22. Spin at 15,000 x g for 15 min at 4 °C.
  23. Discard the supernatant very carefully at this stage since the pellet is not adhered tightly to the tube.
  24. Leave the tubes open in a Class II BSC for 10-20 min for faster drying of the pellet. Do not over dry to avoid difficulty in solubilizing of the pellet.
  25. Resuspend the pellet in 150  $\mu$ l 1x SDS-PAGE sample buffer. You may need to place tubes in a 65 °C water bath briefly to completely resuspend.
  26. If samples turn yellow due to incomplete removal of TCA, add a few microliters of 1 M Tris-HCl (pH 8) until they turn blue again.
  27. Store sample at -70 °C. Thaw before use. 10  $\mu$ l of the sample may be enough for SDS-PAGE and western analysis, although the amount will depend upon the amount of the target protein and the sensitivity of the detection method.

### **Notes**

1. For applications incompatible with TCA precipitations, Centricon Plus-70 centrifugal filter units can be used to concentrate the CF from step B9 as per the manufacturer's instructions. This can be followed by buffer exchange to desired buffers. Care must be taken to use Centricons with membranes of molecular weight cut-off less than the molecular weight of the target proteins to be analyzed in the CF.

## Recipes

### 1. 7H9 complete media (1 L)

7H9 powder	4.7 g
50% glycerol	4.0 ml
20% Tween 80	2.5 ml
Water	to 900 ml

Dissolve 7H9 powder in water and add glycerol and Tween 80

Adjust the amount of water to give a final volume of 900 ml

Add 100 ml of OADC for Mtb or 100 ml of ADC for Msmeg and BCG

Sterilize through 0.22  $\mu$ m filter and stored at 4 °C (The media can be stored for up to six months. If supplemented with additional additives, like antibiotics or salts, the media should be stored as appropriate for the compounds if they are sensitive to light or degradation.)

*Note: Alternatively, autoclave after dissolving 7H9 and glycerol in 897.5 ml water and then supplement with sterile tween-80 and 100 ml of ADC/OADC and store at 4 °C as noted above.*

### 2. Sauton's media (1 L)

Potassium phosphate, monobasic	0.5 g
L-asparagine monohydrate	4.0 g
Citric acid monohydrate	2.0 g
Ferric ammonium citrate	0.05 g
1% Zinc sulfate monohydrate	0.1 ml
Magnesium sulfate heptahydrate	0.5 g
100% Glycerol	60 ml
Water	to 1 L
20% Tween 80	2.5 ml

Dissolve the above components except for the Tween 80 in 900 ml water

Adjust pH to 7 with 5 N NaOH

Make up the volume to 1 L with water

Autoclave, cool, and add 2.5 ml of sterile 20% Tween 80

Stored at 4 °C as mentioned above for 7H9 complete media

### 3. Chelated Sauton's media (1 L)

Potassium phosphate, monobasic	0.5 g
L-asparagine monohydrate	4.0 g
Citric acid monohydrate	2.2 g
Glycerol	60 ml



20% Tween 80 2.5 ml

Water to 1 L

Chelex 100 resin 10 g

Dissolve the components in water

Adjust the pH to 7.4 with 5 N NaOH and make up the volume to 1 L with water

Add 10 g Chelex 100 resin and stir the media for 1-2 days at room temperature

Sterilize through a 0.22 µm filter unit

Dissolve 1 g of MgSO<sub>4</sub>·7H<sub>2</sub>O in 5 ml water and sterilize it by filtration and add to the sterilized media

Stored the media at 4 °C as mentioned above for 7H9 complete media

Defined concentrations of metals like iron, copper and zinc can be added back after chelation.

#### 4. Protein extraction buffer (10 ml)

1 M Tris-Cl (pH 7.5) 0.5 ml

0.5 M EDTA 0.1 ml

20% SDS 0.3 ml

Water 9.1 ml

This can be prepared and stored at 4 °C

Immediately prior to use, add Halt Protease Inhibitor cocktail to a final concentration of 1x and keep on ice.

#### 5. 5x SDS-PAGE sample buffer (50 ml)

0.5 M Tris (pH 6.8) 12.5 ml

SDS 5 g

100% glycerol 25 ml

Bromophenol blue 0.030 g

β- mercaptoethanol 500 µl

Water to 50 ml

Dissolve SDS in Tris buffer and some water, followed by addition of glycerol and bromophenol blue.

Mix and make up the volume to 50 ml with water

Add 500 µl of β- mercaptoethanol

Freeze in aliquots at -20 °C

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