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# Identification of Effectors: Precipitation of Supernatant Material

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1 **Chapter 30**

2 **Identification of effectors: Precipitation of supernatant material**

3 **Running Head:** TCA precipitation

4

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**i. Summary**

Bacterial secretion systems allow the transport of proteins, called effectors, as well as external machine components in the extracellular medium or directly into target cells. Comparison of the secretome, *i.e.* the proteins released in the culture medium, of wild-type and mutant cells provide information on the secretion profile. In addition, mass spectrometry analyses of the culture supernatant of bacteria grown in liquid culture under secreting conditions allows the identification of secretion systems substrates. Upon identification of the substrates, the secretion profile serves as a tool to test the functionality of secretion systems. Here we present a classical method used to concentrate the culture supernatant, based on TCA precipitation.

**ii. Key words:** supernatant, TCA precipitation, secretome

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## 48 **1. Introduction**

49 Bacterial secretion systems are macromolecular machines dedicated to the transport of proteins across the  
50 cell envelope. These secretion systems deliver effectors outside the cell, either in the medium (T1SS,  
51 T2SS, T5SS, T9SS) or directly into target cells (T3SS, T4SS, T6SS) (**1**). Secretion of effector proteins  
52 into the milieu can be observed in these systems and the analysis of secretion supernatant has been widely  
53 used either to identify new secreted effectors or to probe the functionality of secretion systems. For  
54 contact-dependent system such as the T3SS, in vitro secretion in the medium can be observed upon  
55 certain conditions (such as Ca<sup>2+</sup> depletion, acidic pH, etc.) (**2, 3**). If effectors can be predicted by  
56 bioinformatics approaches for several of these secretion systems (*see Chapter 2*), it is not always  
57 possible, and analysis of the content of the culture media, the so-called secretome, using global proteomic  
58 approaches has been widely used to identify secretion system substrates in T2SS (**4-7**), T6SS (**8-10**),  
59 T3SS (**11**) or T9SS (**12**).

60 Upon identification of the substrates, the secretion profile is used to test the functionality of the secretion  
61 system using SDS-PAGE of supernatant fraction followed by Coomassie blue staining or immuno-  
62 staining by Western blot detection of specific effectors or components of the machinery. In some  
63 secretion system, such as T3SS or T6SS, structural external components are released in the milieu upon  
64 secretion and can also be used to test the proper assembly of the system. For example, the Hcp release  
65 assay is widely used to probe the functionality of the T6SS (*see also Chapter 32*).

66 Such analyses of secretomes require to concentrate the dilute solutions that are the culture supernatant or  
67 the biological fluids. This can be achieved using trichloroacetic acid (TCA) precipitation and acetone-based  
68 protocols (**13,14**). Alternative protocols have been proposed, using acetone alone, methanol/chloroform  
69 (**15**), or a combination of pyrogallol red, molybdate and methanol (**16**).

70 Here we detail the most classical assay used to precipitate proteins of bacterial culture supernatant based  
71 on TCA precipitation that is used thoroughly in secretion systems studies. First, cells and supernatant are

72 separated by centrifugation. Cell-free culture supernatant fraction samples are then obtained by further  
73 centrifugation and filtration and subjected to TCA precipitation before analysis by mass spectrometry or  
74 Western blot.

75

## 76 **2. Materials**

77 1. Lysogeny Broth (LB) or the recommended medium to grow the strain of interest in secreting  
78 conditions.

79 2. Trichloroacetic Acid ( $\text{CCl}_3\text{COOH}$ , MW: 163.39, TCA): 100% (w/v) . Add 227 mL of ultrapure water  
80 to a previously unopened bottle containing 500 g of TCA (*see Note 1*). Wear personal protective  
81 equipment and work under a fume hood.

82 3. Sodium Deoxycholate (DOC): 16 mg/mL (Optional, *see Note 2*). Store at room temperature.

83 4. Acetone. Pre-chill before use.

84 5. Refrigerated centrifuge capable of  $21,460 \times g$  or table top centrifuge (*see Note 3*).

85 6. 0.22- $\mu\text{m}$ -pore-size syringe filters (*see Note 4*).

86 7. 2-mL syringe.

87 8. 3 M Tris-HCl, pH 8.8

88 9. SDS-PAGE loading buffer: 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol,  
89 0.01% bromophenol blue.

90 10. Boiling water bath or thermomixer.

91 11. Vortexer

92 12. 2-mL microtubes (Safe-lock) (*see Note 3*)

93 13. Fume hood and personal protective equipment for TCA handling.

94 14. Spectrophotometer to measure absorbance at  $\lambda=600$  nm

95 15. SDS-PAGE and Protein transfer apparatus

96

97 **3. Methods**

- 98 1. Grow a 10 mL bacterial strain culture in the appropriate medium and conditions allowing secretion (*see*  
99 **Note 5** and **Note 6**). Measure the optical density at  $\lambda=600$  nm ( $OD_{600}$ ).
- 100 2. Dispose the culture in 2-mL microtubes (*see Note 7*) and pellet cells by centrifugation at  $6,000 \times g$  for  
101 5 min.
- 102 3. Carefully remove 1.8 mL of supernatant and transfer it in a new microtube and keep it on ice before  
103 performing step 5.
- 104 4. Carefully discard the remaining 200  $\mu$ L of supernatant from the cell pellet obtained in step 3.  
105 (Centrifuge again at  $6,000 \times g$  for 5 min if the cells from the cell pellet started to resuspend). Keep this  
106 total cell fraction pellet on ice before resuspending the pellet in an appropriate volume of SDS-PAGE  
107 loading buffer (The equivalent of 0.2-0.5  $OD_{600}$  units (ODU) / 10  $\mu$ L). Store on ice (or  $-20^{\circ}\text{C}$ ).
- 108 5. Centrifuge the 1.8 mL supernatant fraction obtained in step 3 at  $16,000 \times g$  at  $4^{\circ}\text{C}$  for 5 min. Recover  
109 carefully the supernatant and transfer it in a new microtube. Avoid to recover the remaining cells from the  
110 pellet, if any.
- 111 6. Filter-sterilize the supernatant using a syringe 0.22- $\mu$ m filter and transfer the filtered supernatant  
112 directly in a new microtube. Check the volume (around 1.5 mL). This fraction constitutes the cell free  
113 fraction (*see Note 8*).
- 114 7. Add TCA to a final concentration of 20% (add 375  $\mu$ L of TCA to 1.5 mL of filtered supernatant).  
115 Invert 4 times to mix, vortex and keep on ice for one hour to overnight.
- 116 8. Centrifuge at  $21,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ .
- 117 9. Discard the supernatant as much as possible (*see Note 9*).
- 118 10. Resuspend the pellet in 400-500  $\mu$ L of cold acetone. Vortex.
- 119 11. Centrifuge at  $21,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . Discard the supernatant with a pipet and further on a  
120 paper towel. Leave the tube open at room temperature to dry the pellet (*see Note 10*).

- 121 12. Resuspend the pellet in appropriate buffer for further analysis (such as mass spectrometry) or go to  
122 step 13 for SDS-PAGE analysis.
- 123 13. Resuspend TCA-precipitated pellets of supernatant fractions in an appropriate volume of SDS-PAGE  
124 loading buffer (1 ODU / 10  $\mu$ L). If the TCA-precipitated sample turns yellow, add 1  $\mu$ l (or more) of Tris-  
125 HCl, pH8.8.
- 126 14. Vortex. Heat the samples from step 4 (whole cell fraction) and step 13 (cell free supernatant  
127 precipitated fraction) at 95°C for 10 min. (*see Note 11*).
- 128 15. Analyse whole cells samples and cell free supernatants by SDS-PAGE, followed by Coomassie blue  
129 staining or immunoblot. If performing Western blot, include a control for cell lysis, using antibodies  
130 detecting an internal protein. Alternatively, check the Coomassie or Silver staining profile.

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#### 133 4. Notes

- 134
- 135 1. For a safe and easy preparation, avoid weighting out the TCA crystalline powder, as it becomes  
136 easily syrupy upon contact with air moisture. The TCA solution must be kept in dark glass bottle.  
137 It is very corrosive and should be handle with care with suitable protection. Do not use plastic  
138 containers.
  - 139 2. DOC may be used as a carrier to assist protein precipitation. If using DOC, add the DOC stock  
140 solution at the final concentration of 0.16 mg/mL to the cell free fraction obtained in step 6,  
141 vortex and leave on ice for 30 min, then proceed to TCA precipitation as described in step 7.  
142 DOC should be washed out with further acetone washing steps (repeat steps 10-11 three times).  
143 However, it could be a problem with further mass spectrometry analysis.
  - 144 3. Table top centrifuge at maximum speed may be sufficient, however we generally use higher  
145 speed. TCA resistant tubes should be use, such as Eppendorf tubes (check with your manufacturer  
146 for tube compatibility).

- 147 4. In principle, any 0.22  $\mu\text{m}$  filter may be used. However, we had experience with a secreted protein  
148 that was retained on PVDF filters, so we moved to Polyether sulfone (PES) filters. Be aware that  
149 material of the filter may be of importance.
- 150 5. A "non secreting strain" should be used as a control, such as a mutant in a core component, or the  
151 ATPase energizing the assembly of the secretion machinery or the substrate transport.
- 152 6. You must find conditions where secretion can be detected in vitro. As effectors can be secreted in  
153 low levels, high sensitivity mass spectrometry methods may be required (**10**). In case the  
154 secretion system is not produced in laboratory conditions, native endogenous promoter(s) may be  
155 swap by an inducible promoter (*Ptac*, *Plac*, *PBAD*, etc.) to artificially induce the expression of the  
156 secretion system (**17**).
- 157 7. A 5-10 mL of culture is generally sufficient. We usually transfer 2 mL of supernatant in 2-mL  
158 microtubes, leading to the recovery of 1.5 mL of cell free supernatant. An equivalent of 1 OD<sub>600</sub>  
159 unit will be loaded on the gel for supernatant fractions analysis. For scale up experiments, you  
160 will have to use tubes with bigger volume compatible with high speed spin and that are resistant  
161 to TCA. Appropriate 50-mL tubes (polyether) may be used, check first with your manufacturer  
162 for TCA compatibility.
- 163 8. At this stage, for bacteria producing high levels of vesicles (*e.g* for T9SS in Bacteroidetes), an  
164 additional ultracentrifugation (30,000  $\times$  g for 4 h at 4°C) will allow to separate vesicles from  
165 vesicle-free supernatant (**12**).
- 166 9. Check the orientation of the microtube before the centrifugation step, as the pellet is not always  
167 visible at this stage.
- 168 10. You may use a vacuum concentrator (SpeedVac or equivalent) for 10 min to evaporate the  
169 acetone. However, pellets may be more difficult to resuspend if too dry, and this step may  
170 decrease recovery of the samples.
- 171 11. In some cases we observed that an additional freezing at -20°C in SDS-PAGE loading buffer  
172 helps resuspension of TCA precipitates.

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