**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

* We have proofread the manuscript as requested.

2. Please provide an email address for each author.

* These have been added to the first page.

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Percoll" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

* We have changed the text throughout to refer to “density” gradients most of the time, including in the title, and only mention Percoll in the specific protocol steps where it is used. We have added a sentence about Percoll and what it is in the discussion as suggested by Reviewer 1, but state that other substances could be used to establish the gradient if they fit the criteria of low toxicity and viscosity (line 374).

4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

* Revised as requested.

5. Please revise the protocol (2.1, etc.) to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

* We have restructured the protocol instructions to separate the imperative instructions from the other considerations, and have added text about safety considerations (lines 95-102, 136, 182, 267).

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:  
1.1: Please specify the bacterial strains tested in the protocol and the incubation temperature used.  
1.2.2: Please specify centrifugation parameters. What container is used in this step? What volume  
3.8.2: What volume of fresh Percoll gradient is needed in each tube?  
What happens after centrifugation, discard the supernatant? Please specify throughout.

* Changed as requested.

7. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

* We have attempted to rewrite the discussion to cover these points explicitly, though we do not wish to repeat material in the introduction where we make the primary case for the value of this method.

8. A minimum of 10 references should be cited in the manuscript.

* We have added more citations.

9. Please remove reference #8. Manuscripts that are in preparation or under review should not be listed as reference.

* This methods article is a counterpart to reference 8, which is currently under revision for *mBio*. We previously discussed this manuscript with Lyndsay Troyer and Indrani Mukherjee at *JoVE* and asked for the *JoVE* article to only be published after this paper, which they said was fine (we can forward on the relevant emails if you need). Therefore, this reference will be updated once our other manuscript is accepted, which we hope will be very soon.

**Reviewers' comments:**

**Reviewer #1:**  
Manuscript Summary:  
This manuscript describes the physical separation of bacteria based on the level of Capsule production using a percoll gradient. The abstract and introduction are clear and convincing that the need exists for a better method to physically separate capsule producers from non-producers in Klebsiella and other species. The background given is sufficient, although a sentence about what percoll is and how it works might be helpful.  
The methods section could be tidied up, as a good portion of it is unclear for a user who has never done this before. I am sure the video would help to clarify, but since I am just reviewing the written method, I have some specific questions and comments below for the written text.  
  
Major Concerns:  
Line 93, Step 1.2.2: This seems like a critical step where issues/problems could arise. You have previously mentioned (in the introduction) that some strains, especially those with altered capsule production, do not pellet uniformly. Therefore this step seems critical as you may very well miss the population you are actually looking for because your mutant does not pellet well or it pellets differently than the rest of the population (especially true when you are talking about identification of mutants in a mixed library). Can this step be avoided (e.g. by a filtration and wash step, or can you just keep the cells in growth media instead of resuspending in PBS?)

* We have added additional description to this step (now line 123) to make it clear that bacteria should not be removed (even if this means leaving behind supernatant), and that for cells that do not pellet well then using cultures directly is an option.

Line 139, Method 1 and Line 157, Method 2  
Please mention what these two methods are used for. Why are you doing Method 1: top to bottom and Method 2: bottom to top? I'm not sure if I have misunderstood this section. I.e. the figures do not indicate these two different methods?

* We have clarified that these are simply two routes to achieve the same end – in our lab, some people prefer to prepare density gradients with a needle starting from the least dense fraction, while others prefer to use a pipette and work from most dense to least dense. The method with the pipette has been relegated to an alternative protocol at the end (Step 7, line 317). Thank you to the reviewer for pointing out that this was not clear.

Minor Concerns:  
Line 93, Step 1.2.2:  
\*Please specify speed and time of centrifugation here.

* Added this information.

Line 98, Step 2.1:  
\*"Exact concentrations needed" should be clarified to: "exact concentrations of percoll needed". Upon first read it was not clear if you were talking about concentrations of percoll or bacteria.  
\*Please specify the difference between a mini gradient and a regular one. I.e. I think (?) that the mini gradient just has one concentration of percoll and the standard gradient contains three, but this is not specified in the text at this point. Also, please state the volumes used in mini and standard gradients.  
\*One might include that a hyper capsulated control as well as non-capsulated could be used.  
\*Is there any special requirements for making up different concentrations of Percoll? Just mix with PBS? Do you sterilize the Percoll?

* We have clarified all the above points.

Line 106, Step 2.1.1:  
\*What is the total volume of Percoll needed per concentration/assay? It should be stated here.

* This will depend on the number of strains of interest, however, we have added precise volumes needed per sample for each step.

Line 113-120, Step 2.2 and 2.2.1:  
\*This needs to be changed. By step 2.2.1, as it is written, the bacterial cells have already been applied to the top of the percoll (step 2.2), so it is too late to talk about doing it extremely slowly, etc. I would recommend making Step 2.2 a header that says: applying bacteria to percoll, or just combining these steps into a single clear one.

* Changed as requested.

Line 123, Step 2.2.3.  
A Figure or schematic associated with this step could aid in understanding. Like Fig Bi), but for the mini-gradient.

* We have pointed the reader to Figure 2A, which shows the output of two mini-gradients like the ones used for optimisation. We will demonstrate the use of mini-gradients for optimisation in the video.

Line 136, Step 3.2  
Specify how many percoll concentrations one will use (three?) in a single tube. As it is, it is hard for me to figure out if the mini-gradient has one concentration of percoll while the standard gradient has three concentrations. Again, you can specify here the total volume needed per strain being tested.

* We have changed this as requested.

Line 141, Step 3.2.2:  
\*You say use a "blood tube", but this is not listed in your materials. Do you mean a "5ml polyproleyn (spelling not correct: should be polypropylene) round bottom tube"? I think the use of the term "blood tube" is confusing, just say 5ml polypropylene round bottom tube.  
\*This step could be re-written to be more clear ◊ maybe to "3.2.2 Pipette 1 ml of the most dilute percentage Percoll being used into a 5 ml polypropylene round bottom tube." An additional note here is needed, something like: This layer will form the top layer of the percoll gradient. Subsequent layers of more concentrated percoll will be added below this layer, using a needle, so as not to disrupt the layers. The top layer is the most dilute layer.

* Changed as requested, thank you for the suggestion.

Line 143, Step 3.2.3  
"Using a 1 ml disposable syringe with a 2-inch needle attached, take up 1 ml of the next most concentrated percentage Percoll dilution."  
Why is a needle used and not a pipette tip? There is no reason given as to why you are using a needle vs pipette here. Assume as it disrupts the gradient less.

* This is to cause less disruption of the gradient – we have now stated this in the text.

Line 145, Step 3.2.3  
"This can also be done with a 1 ml attached to the needle using Parafilm."  
This is not a good idea from a health and safety standpoint. Needles should only be used with the appropriate syringe luer lock system. You risk a needle stick doing this. I recommend to remove this sentence.

* Removed as suggested.

Line 168, Step 3.4  
By step 3.4.1, as it is written, the bacterial cells have already been applied to the top of the percoll (step 3.4), so it is too late to talk about doing it extremely slowly, etc. I would recommend making Step 3.4 into a header that says: applying bacteria to percoll, or just combining these steps into a single clearer one.

* We have changed this and refer to the method in Step 2.2.

Line 168, Step 3.4  
Are you applying bacteria to the Method 1 (top to bottom) or Method 2 (bottom to top) gradient (or both)? Again, I am not sure I understand the method 1 vs 2.

* Gradients made by either method are the same, this is now made clear to the reader.

Line 177, Step 3.5  
"Blood tube adapter" should be changed to "2.6 - 7ml tube adapter", as listed in your materials.

* Changed as requested.

Line 185, Step 3.7.  
Does one need to be careful not to disrupt the percoll gradient here? Should one use a needle to remove the bacteria?

* We have found that it is easy not to disrupt the gradient when removing fractions sequentially from the top using a pipette. A needle could be used when only a lower fraction is needed and this is now stated in the text.

Line 194-209, Step 3.8 to 3.8.3  
It is not clear to me why is it necessary to re-purify the sample? It seems sufficient to just grow the fraction and directly do the gDNA extraction? Have you had trouble at this step, if so, a reader might want to know this re-purification is a critical step.

* The repurification is to reduce the carryover of cells from higher fractions (i.e., more highly capsulated cells) when extracting low-abundance lower fractions. This is not always necessary but can be useful, and we have clarified this in the text.

Line 196: This sentence is not clear and needs to be clarified: "Transfer cells from the low-abundance fraction 5 ml liquid media and grow."

* Changed as requested.

Line 204: "This should be the concentration from just above the original… " is this from Method 1 or Method 2? IS this concentration more concentrated or less?

* We have rewritten this to make it clearer.

Figures:  
\*Please modify Fig 1 Bi) to have increasing levels of shading for A, B, and C to indicate the density of the percoll.

* We have changed this, thank you for the useful suggestion.

\*Include a figure for Method 1 and 2 (line 139 and 157) gradients top to bottom and bottom to top.

* Our attempts to make a figure to illustrate the gradient setup clearly were not successful – however, we plan to demonstrate both of these methods in the video, and now that method 2 is presented as an alternative method at the end of the paper we feel readers are not likely to be confused about these methods.

\*Indicate in the Figure 1 which fraction contains the section with bacterial cells containing more or less capsulated cells. (e.g. in Fig 1Bi), you can modify the labelling to be something like: Top/cap++, middle/cap+ , bottom/cap-

* Changed as suggested.

\*Figure 1 B ii) more description of what this is needed. An example of what is meant by a complex sample and what the contaminants are would be helpful and should be expanded on.

* Changed as suggested. We use the example of a mixed bacterial culture containing capsulated and non-capsulated bacteria.

\*Figure 2B i) is not clear. A new picture is needed here or delete this figure, as it is not helpful. The background should be white or at least a uniform color, and tube not in a rack.  
\*Figure 2 D) The background should be white/black or a uniform color.

* We have replaced both of these photos with ones using a black background.

**Reviewer #2:**  
Manuscript Summary:  
The authors have developed a suitable method to separate bacteria by capsule amount, using a discontinuous Percoll gradient. While simple, this method is of importance in virulence studies because there is no widely used protocol for sorting bacteria based on how much capsule they produce. As mentioned by the authors, this method can be used to compare capsule amounts, to isolate mutants with altered capsule production, and to purify capsulated bacteria from complex samples.  
  
Major Concerns:  
The main concern is that results of this semi-quantitatively method will depend on the bacterial species used. In this paper authors only illustrate the method using K. pneumonia. Illustrating the effectiveness of the method using another capsule producing bacteria such as Pseudomonas aeruginosa, Streptococcus pneumoniae, Haemophilus influenzae and Neisseria meningitidis would have been highly desired.

* We previously applied this method to *S. pneumoniae* and have included this result as an additional figure. This method has also been used successfully for *Pasteurella multocida* by our colleague (Thomas Smallman, personal communication). We envisage that this method will be useful for the majority of bacteria with true surface-attached capsules, although we have not yet tested a wide range of species. We include this caveat and ways to test the effectiveness of this method in the discussion section.