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Separating bacteria by capsule amount using a discontinuous density gradient

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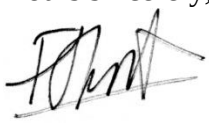
Dear Dr Bajaj,

We wish to submit the revised version of our manuscript: “Separating bacteria by capsule amount using a discontinuous density gradient”.

Our revised manuscript is marked up with tracked changes and comments. We have made almost all of the requested changes in order to improve the clarity of the manuscript and its alignment with JoVE style. There are two suggested changes that we have not made, following discussions with Nam Nguyen at JoVE. These are that we have retained some text in the protocol that was added at the request of Reviewer 1, and that we have not eliminated the use of the word “Percoll” in the protocol (though we have reduced its use). We feel our manuscript is improved following editorial review, in addition to the previous round of peer and editorial review, and hope that it now aligns with the JoVE standard. Please do not hesitate to contact us with any other queries or suggestions.

Thank you for the time you have taken to consider our manuscript, and we look forward to receiving your final decision.

Yours sincerely,



Dr Francesca L. Short

TITLE:

Separating Bacteria by Capsule Amount Using a Discontinuous Density Gradient

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KEYWORDS:

Discontinuous density gradient, capsule, TraDIS, hypermucoidy, *Klebsiella*

SUMMARY:

We demonstrate the use of discontinuous density gradients to separate bacterial populations based on capsule production. This method is used to compare capsule amount between cultures, isolate mutants with a specific capsule phenotype, or to identify capsule regulators. Described here is the optimization and running of the assay.

ABSTRACT:

Capsule is a key virulence factor in many bacterial species, mediating immune evasion and resistance to various physical stresses. While many methods are available to quantify and compare capsule production between different strains or mutants, there is no widely used method for sorting bacteria based on how much capsule they produce. We have developed a method to separate bacteria by capsule amount, using a discontinuous density gradient. This method is used to compare capsule amounts semi-quantitatively between cultures, to isolate mutants with altered capsule production, and to purify capsulated bacteria from complex samples. This method can also be coupled with transposon-insertion sequencing to identify genes involved in capsule regulation. Here, the method is demonstrated in detail, including how to optimize the gradient conditions for a new bacterial species or strain, and how to construct and run the density gradient.

INTRODUCTION:

Many bacterial species produce a polysaccharide capsule, which protects the bacterial cell from various physical stresses and from recognition and killing by the immune system. In *Klebsiella pneumoniae*, capsule production is an absolute requirement for infection[1,2]. *K. pneumoniae*

capsule mediates resistance to antimicrobial peptides, resistance to complement-mediated killing, prevention of phagocytosis, and suppression of the innate immune response[3]. Excess capsule production is associated with increased virulence and community-acquired (rather than nosocomial) infections[4].

A range of quantitative and qualitative tests are available to investigate capsule production. For *Klebsiella* species, these include the string test[5], in which a toothpick touched to a colony is pulled upwards and the length of the string produced measured, and the mucoviscosity assay[6], which involves the slow centrifugation of a culture followed by measuring the optical density of the supernatant. These methods are simple and quick, but lack sensitivity when used on classical *Klebsiella* strains rather than capsule overproducing strains. Another method of capsule quantification is the uronic acid assay, which is technically challenging and requires the use of concentrated sulfuric acid[1]. Finally, capsule is visible directly by microscopy (**Figure 1A**). Of these methods, only microscopy allows the user to observe different capsulation states within a single population, and none of these methods enables the physical separation of capsulated and non-capsulated bacteria.

Density-based separations by gradient centrifugation are routinely used in cell biology to purify different eukaryotic cell types[7], but are rarely used in microbiological research. The mucoviscosity assay for *Klebsiella* is based on the observation that highly capsulated bacteria take more time to pellet by centrifugation, and we reasoned that this may be due to reduced overall density of capsulated cells. The method shown here was developed to separate *K. pneumoniae* populations physically by capsule amount, using density gradient centrifugation (**Figure 1**). This method was applied successfully to *Streptococcus pneumoniae*, indicating that it is applicable to other bacterial species. Density-gradient separation of a saturated transposon mutant library coupled with transposon-insertion sequencing (density-TraDISort) has been used to identify genes involved in the capsule production and regulation[8]. Similarly, this method was used in conjunction with random-prime polymerase chain reaction (PCR) of individual colonies to isolate non-capsulated *K. pneumoniae* mutants. This method can also be used for rapid comparisons of capsule production between different populations and conditions, or to purify capsulated bacteria from complex samples (**Figure 1B**). Finally, there is the option to assay other phenotypes that affect density, such as cell size or aggregation.

This manuscript demonstrates how to optimize the procedure for a new bacterial species or strain and demonstrates the construction and running of a discontinuous density gradient to separate hyper-capsulated, capsulated and non-capsulated bacteria.

PROTOCOL:

Note: Ensure that any risk assessments applicable to the bacterial strains are adhered to when culturing and handling samples. Be aware that setting up too many gradients at one time can lead to musculoskeletal disorders due to the pressure on joints from the slow pipetting involved. Plan work and take precautions to avoid injury.

1. Preparation of Bacterial Strains or Mutant Libraries

1.1. Streak out the strains to be tested on appropriate agar plates. These are stock plates for the experiment.

1.1.1. Incubate the plates overnight at the desired temperature to achieve single colonies. For this experiment, culture *K. pneumoniae* (NTUH-K2044 and ATCC43816 strains) on Luria broth (LB) agar at 37 °C, and *S. pneumoniae* (23F wild type and 23F Δ*cps*) on blood agar in a humidified candle jar at 37 °C.

1.2. Pick a single colony from a stock plate (step 1.1) to inoculate 10 mL of appropriate broth using a sterile loop or cocktail stick. For screening of random mutant libraries, inoculate the broth with 10 µL of the random mutant library stock (TraDIS Library).

1.2.1. Incubate *K. pneumoniae* strains in low salt LB media at 37 °C with shaking, and *S. pneumoniae* strains in brain heart infusion (BHI) media, statically at 37 °C.

1.2.2. Transfer the overnight culture to a 15 mL tube and centrifuge in a bench-top centrifuge for 10 min at 3,200 x g in swing out buckets with 15 mL tube inserts and aerosol tight lids.

1.2.3. Discard the supernatant and resuspend the pellet in 2 mL of 1x phosphate-buffered saline (PBS).

Note: Dispose of supernatant *via* the appropriate liquid biological waste route in the laboratory. The purpose of the centrifugation and resuspension steps (1.2.2 and 1.2.3) is to concentrate the bacteria for easy visualization on the gradient. Bacterial cultures can be loaded directly onto the gradient if preferred. Heavily capsulated strains may not form a tight pellet. If this occurs, remove as much supernatant as possible without removing any bacterial cells, add 1x PBS to a final volume of 5 mL and resuspend the pellet. Continue the protocol with step 1.2.5.

1.2.4. For non-mucoid strains, go to step 2.

1.2.5. Centrifuge the tubes as described in step 1.2.2.

1.2.6. Discard the supernatant and resuspend the pellet in 2 mL of 1x PBS. Cells are now ready to use.

Note: The density of the bacterial cell suspension is not critical but needs to be sufficient for visualization in the gradient. A minimum OD_{600} (optical density at 600 nm) of 4 is suggested.

2. Preparation of Gradient Dilutions and Mini-gradient Test

2.1. Prepare gradient dilutions.

Note: Exact concentrations of density gradient medium (*e.g.*, Percoll) needed in the density gradients to achieve good separation will differ depending on the bacterial strain and growth conditions used. Mini-gradient tests are performed first, to identify the concentrations that will give the best separation. These comprise 500 µL of a single dilution in a 2 mL tube. If bacteria will be extracted from the gradient and subcultured for downstream applications, these steps should be performed under aseptic conditions.

2.1.1. Combine density gradient medium with 1x PBS to make the density gradient dilutions. Make dilutions of 20%, 30%, 40%, 50%, 60%, 70%, and 80% (*e.g.,* 2 mL of density gradient medium plus 8 mL of 1x PBS = 10 mL of 20% density gradient medium).

2.1.2. Aliquot 500 µL of the 20% gradient dilution into a 2 mL tube for each strain to be tested (*e.g.,* 4 strains = 4 tubes containing 500 µL of 20% gradient dilution).

2.1.3. Repeat step 2.1.2 for the rest of the gradient dilutions.

2.2. Apply bacteria to the gradient.

2.2.1. Apply 100 µL of bacterial cells prepared in steps 1.2.3 and 1.2.6 to the top of each gradient dilution following the steps below.

2.2.1.1. Take up 100 µL of cells using a 200 µL pipette and place the pipette tip on the side of the tube just below the meniscus of the mini-gradient.

2.2.1.2. Aspirate the bacterial cells onto the gradient extremely slowly so that they form a layer on the top of the gradient, without any mixing of the interface.

2.2.1.3. Repeat steps 2.2.1.1-2.2.1.2 for all strains to be tested.

2.2.2. Using a fixed angle rotor with an aerosol tight lid, centrifuge the prepared tubes in a microcentrifuge for 10 min at 8,000 x g.

2.2.3. After centrifugation, transfer the tubes to a rack to visualize the minimum gradient dilution required to retain cells just above the gradient layer following centrifugation.

2.2.4. If the results are not clear, repeat the mini-gradient test with increments of 5% density gradient medium dilutions above and below the concentrations defined in step 2.1.1 (*e.g.,* 25% and 35% dilutions should be tested if the result at 30% is ambiguous).

Note: See **Figure 2A** for typical results at a single density gradient medium dilution.

2.2.5. Use the results from steps 2.2.3-2.2.4 to determine the ideal gradient dilutions to use to separate cells in larger-scale discontinuous gradients.

3. Preparation of Cells for the Main Experiment

3.1. Prepare fresh overnight cultures by inoculating 10 mL of appropriate broth as described in step 1.2.

3.1.1. Incubate the cultures overnight in appropriate conditions as described in step 1.2.1.

3.1.2. Pellet the overnight culture as described in step 1.2.2.

3.2. Wash the cells as described in step 1.2.3.

3.3. Discard the supernatant and resuspend the pellet in 1 mL of 1x PBS. If the strain is mucoid and does not pellet easily, resuspend the pellet in up to 2 mL of PBS or residual media. Cells are now ready to use.

4. Preparation of Discontinuous Density Gradients

Note: An alternative method of gradient preparation from bottom (most concentrated) to top (least concentrated) using a pipette is described in step 7.

4.1. Pipette 1 mL of the most dilute density gradient dilution into a 5 mL polypropylene round bottom tube to form the top, most dilute, layer of the gradient.

Note: Subsequent layers of more concentrated gradient dilutions will be added below this layer using a needle, so as not to disrupt the layers. A minimum of two and maximum of three gradient layers of 1 mL each are recommended for robust separation and easy visualization of bacteria.

4.1.1. Using a 1 mL disposable syringe with a 1.5-inch needle attached, take 1 mL of the next most concentrated density gradient medium dilution into the syringe. Avoid taking up any air, as bubbles can disrupt the gradient layers.

4.1.2. Place the needle end at the bottom of the tube containing the first layer. Aspirate the syringe contents very slowly to avoid mixing the interface.

Note: The interface of the two dilutions will rise as the more concentrated gradient dilution is added. Observe the interface by holding it up to light or an outside window. If no distinct interface is observed, discard the gradient and start again.

4.1.2.1 Remove the needle from the gradient very gently so as not to disturb the interface between the different gradient dilutions. Place the tube in a suitable rack to keep it upright.

4.1.3. If using three different dilutions, repeat steps 4.1.1-4.1.2.1 so that the densest dilution is at the bottom. If the gradient has been constructed successfully, there will be three distinct layers with no mixing at the interfaces.

5. Adding Prepared Cells to Gradients and Separation by Centrifugation

5.1. Add 500 µL of prepared cells from step 3.3 to the top of the gradient in the tube very slowly and without mixing the interface, as described in step 2.2.

5.2. Place the tubes in tube adapters and weigh the combined adapters and tubes to ensure they are balanced.

5.2.1. Place the tube adapters in a fixed angle rotor within a bench-top centrifuge. Centrifuge for 30 min at 3,000 x g.

5.2.2. After centrifugation, carefully remove the tubes, place them in a suitable rack, and photograph the results as a record.

5.3. Recover the bacterial fractions for uronic acid quantification or DNA extraction by following step 6.

5.3.1. If downstream applications are not required, dispose of the samples/gradients *via* the appropriate liquid biological waste route in the laboratory.

Note: It is important to validate the separation for new species/strains of bacteria. Individual fractions from the gradient should be examined by microscopy, by uronic acid assay (step 8), or another suitable quantitative assay to confirm the capsule phenotype of each fraction. If the aim is to separate based on aggregation or cell size, independent appropriate assays should be used.

6. Recovering Sample Fractions and Optional Outgrowth Step

6.1. Recover the fractions from a gradient by removing and discarding any liquid from the top dilution if no bacterial fraction is present within that layer.

6.1.1. To remove the top fraction, use a P200 pipette and gently pass the pipette tip through the gradient to the fraction, and take up the fraction. Place the fraction into a 1.5 mL tube and label appropriately.

6.1.2. To recover further fractions, still using the P200 pipette, insert the tip *gently* through the gradient to the fraction and recover the fraction to a fresh 1.5 mL tube. Remove excess gradient as the fractions lower down the gradient are accessed.

6.1.3. If DNA extraction from a fraction containing very low cell numbers is required (*e.g.*, for density-TraDISort), subculture the fraction by following the protocol from step 6.2 for optional outgrowth to obtain more cells.

Note: There will be a low level of carryover from cells at the top of the gradient into lower fractions, as fractions are removed from top to bottom. Minimize this by working carefully so as not to mix the gradient, by using a needle to remove lower fractions, and by performing re-purification of very low abundance samples where carryover may affect results.

6.2. Transfer cells from the low-abundance fraction recovered in steps 6.1.1-6.1.2 into 5 mL of appropriate liquid media. Place in an incubator and grow for 2 h at 37 °C.

6.2.1. After 2 h of growth or when the sample has reached an OD_{600} of 1, transfer the culture to a 15 mL tube. Centrifuge the tube for 10 min at 3,200 x g in a centrifuge with swing out buckets and aerosol tight lids. Discard the supernatant

6.2.2. Resuspend the cell pellet in 1 mL of 1x PBS.

6.3. Prepare a fresh single-concentration density gradient in a 5 mL polypropylene tube. Use the gradient concentration from just above the location of the fraction in the original gradient (*e.g.*, for purification of the Δ*slyA* mutant shown in **Figure 2D**, 15% density gradient medium would be used).

Note: This re-purification step is optional.

6.3.1. Apply the cells to the top of the gradient and centrifuge as described in steps 2.2 and 5.1.

6.3.2. Remove the tubes from the centrifuge and place in an appropriate rack. Photograph the gradient and recover the fraction for DNA extraction as described in 6.1-6.1.2.

Note: The sample is now ready for DNA extraction or other downstream applications.

7. Alternative Method for Gradient Preparation from Bottom (Most Concentrated) to Top (Least Concentrated) Using a Pipette

7.1. Use the gradient dilutions determined in step 2.2.3. Using a 1000 µL pipette, add 1 mL of the densest gradient dilution to a 5 mL tube.

7.1.1. Using a 200 µL pipette, add 200 µL of the next dense gradient dilution very slowly to the top of the layer in the tube, so as not to mix the interface. Add another 200 µL and then the final 600 µL. Adding multiple smaller volumes gives more control over pipetting speed and prevents mixing of the interface. If the interface mixes, discard and start again.

7.1.2. Repeat step 7.1.1 with the third, least dense gradient concentration if three dilutions are being used. The tube should now have gradients of either 2 mL or 3 mL depending on the results from step 2.2.3.

7.1.3. Go to step 5 of the protocol to add cells and continue the experiment.

8. Measurement of Capsule Amount by Uronic Acid Assay

8.1. Adjust the OD_{600} of each recovered fraction to 4.0 by dilution with PBS. Proceed with quantification of uronic acids as previously published[1].

REPRESENTATIVE RESULTS:

Representative results are shown in **Figure 2**. The exact result to expect will depend on the bacterial species, the set-up of the density gradients, and whether the user is examining a single strain or a pool of mutants. Most strains will migrate to a single location within a gradient, as shown in **Figures 2A** and **2D.** Applying the method to a bacterial mutant library will give rise to a major band above the gradient, a less dense band distributed through the uppermost layer of the gradient, and a minor acapsular fraction at the bottom (**Figure 2B**). These fractions differ in capsule amount as shown by an assay for uronic acids (**Figure 2B**). Transposon insertion sequencing of individual fractions results in clear localization of specific mutants within different gradient fractions, as shown for the capsule biosynthesis locus of *K. pneumoniae* ATCC43816 (**Figure 2C**). Representative results for pure cultures of *K. pneumoniae* NTUH-K2044 and *S. pneumoniae*, and different capsule biosynthesis or regulatory mutants, are shown in **Figure 2D**.

FIGURE LEGENDS:

Figure 1: Schematic of the density centrifugation method to separate bacteria based on capsule, and its applications. (**A**) An electron microscopy image of a capsulated *Klebsiella pneumoniae* cell. The capsule is visible as a dense layer on the outside of the cell. (**B**) Applications of density centrifugation to the study of capsulated bacteria. (**Bi**) Density separation can be used to generate high-, low-, and no-capsule fractions of a transposon mutant library and followed by transposon insertion sequencing to define genes that influence capsule production. (**Bii**) Purification of capsulated bacteria from a complex sample. (**Biii**) Use of density-based separation for rapid comparisons of capsule amount between samples. This method also allows the visualization of heterogenous capsule production in bacterial populations, as shown in (**Biii**).

Figure 2: Representative results. (**A**) Example of the output from mini-gradient tests. Two different *K. pneumoniae* strains were centrifuged on 1 mL of 15% density gradient medium. The hypermucoviscous NTUH-K2044 strain is retained above the density gradient medium layer, while ATCC43816 (which makes less capsule) migrates to the bottom of the layer. (**Bi**) Use of a density gradient to separate a transposon mutant library into three fractions. Note that the bottom fraction contains a low proportion of mutants and is not visible on this picture. (**Bii**) Validation of different capsule amounts in the top, middle, and bottom fractions using an assay for uronic acids. Cells from the top, middle, and outgrown bottom fractions were isolated and resuspended in PBS to an OD_{600} of 4, then capsule polysaccharides extracted and uronic acids measured[1]. (**C**) Example density-TraDISort results. Mutation locations identified are shown by blue lines above the chromosome diagram. Mutants lacking capsule can be identified as those that are present in the input library but are depleted in the top fraction while being enriched in the bottom fraction, as shown here for the capsule biosynthesis locus. (**D**) An example of using

density gradient centrifugation to compare capsule amount between wild type and mutant strains of *K. pneumoniae* NTUH-K2044 and *S. pneumoniae* 23F.

DISCUSSION:

Capsule is an important virulence factor in many bacterial species including *K. pneumoniae*[3], *Streptococcus pneumoniae*[9], *Acinetobacter*[10], and *Neisseria*[11] species. Although various methods exist for quantification and visualization of bacterial capsules, at present there is no widely used method to physically separate capsulated and non-capsulated cells. In this article, we have demonstrated a robust method for capsule-based separation of bacterial populations, with multiple potential applications in conjunction with different upstream or downstream protocols.

The presence of a surface capsule can reduce bacterial cell density, which allows separation by density gradient centrifugation (**Figure 2D**). We have validated this method in *K. pneumoniae* NTUH-K2044[12] and ATCC43816[13] as well as in *Streptococcus pneumoniae* 23F[14] and its Δ*cps* mutant[15]. This method uses Percoll[16] as the main constituent of the density gradient, which is a suspension of coated colloidal silica particles that has low viscosity and no toxicity towards bacteria — in principle, other substances meeting these criteria could be used to establish the density gradient.

It can be challenging to ensure that layers of different density do not mix when constructing density gradients, and if mixing does occur, the separation method will not give clean results. We have included two alternative methods for pouring the gradients, using a needle or a pipette — both are effective, and which method to use is simply a matter of preference. For all steps that involve pipetting a substance (either a bacterial suspension, or a more dilute gradient layer) above a gradient layer, pipetting multiple aliquots of smaller volumes can make it easier to achieve a sharp interface without any mixing of layers.

A limitation of this protocol is that its performance with other bacterial species cannot be guaranteed. Therefore, it is critical when examining a new bacterial species or strain to validate the density-based separation using an additional, independent capsule quantification method. Visualizing the bacteria present in each fraction by microscopy with appropriate capsule stains is a reliable method for which detailed protocols are available[17]. Alternatively, capsules containing uronic acids (such as those of *Escherichia coli* and *K. pneumoniae*) can be quantified by a specific assay as shown in **Figure 2B**[1]. The centrifugation-based mucoviscosity test is not suitable as an independent validation method, as this assay also depends on the density of the bacterial cells.

Another limitation of this method is that capsule production is very sensitive to culture conditions, and even small changes to growth medium, temperature, or aeration may affect the results of this assay. To minimize this issue, researchers can use a defined growth medium or a batch-consistent complex medium, keep all other growth parameters identical between experiments, and include appropriate control strains to enable the interpretation of unexpected results. Some bacterial capsules are fragile and can shear away from the cell when cultures are pipetted. To avoid the shearing of capsules, cultures should be centrifuged and resuspended no more than twice during preparation for loading on the gradient. If loss of capsule during

concentration of the cultures remains problematic, bacterial cultures can be applied to a density gradient directly, with a larger volume of bacterial suspension added if necessary for visualization.

Future applications of this method are to apply it to other bacterial species, and to use this separation in conjunction with different upstream and downstream technologies. In addition to density-TraDISort[8], we suggest that density gradient separation of capsulated bacteria could be used for isolation of mutants with altered capsule, for purification of capsulated cells from mixed cultures or complex samples, and for rapid profiling of capsule production in multiple strains. Finally, this technology could be used to examine other bacterial phenotypes such as aggregation.

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DISCLOSURES:

The authors have no financial interests to disclose.

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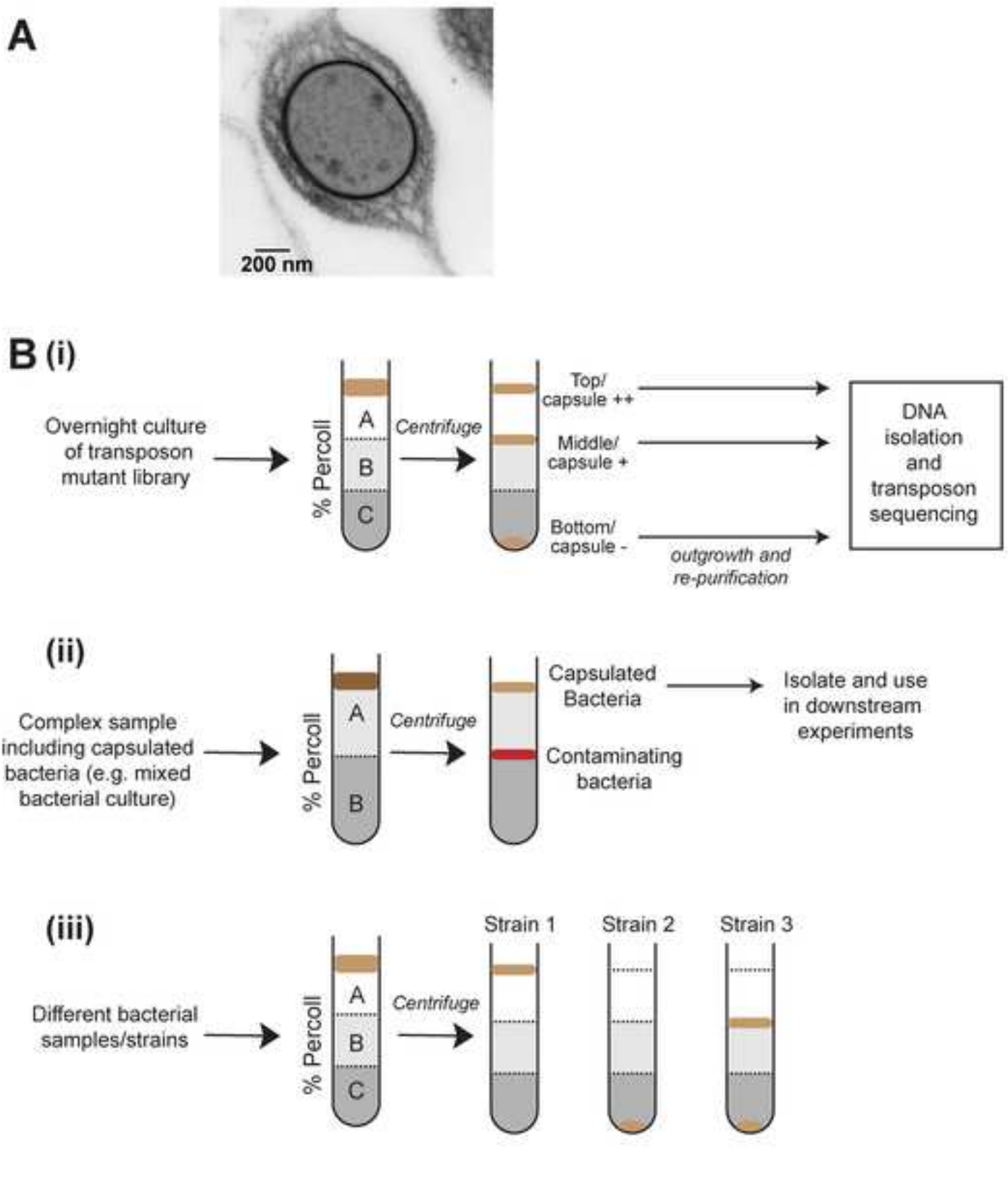
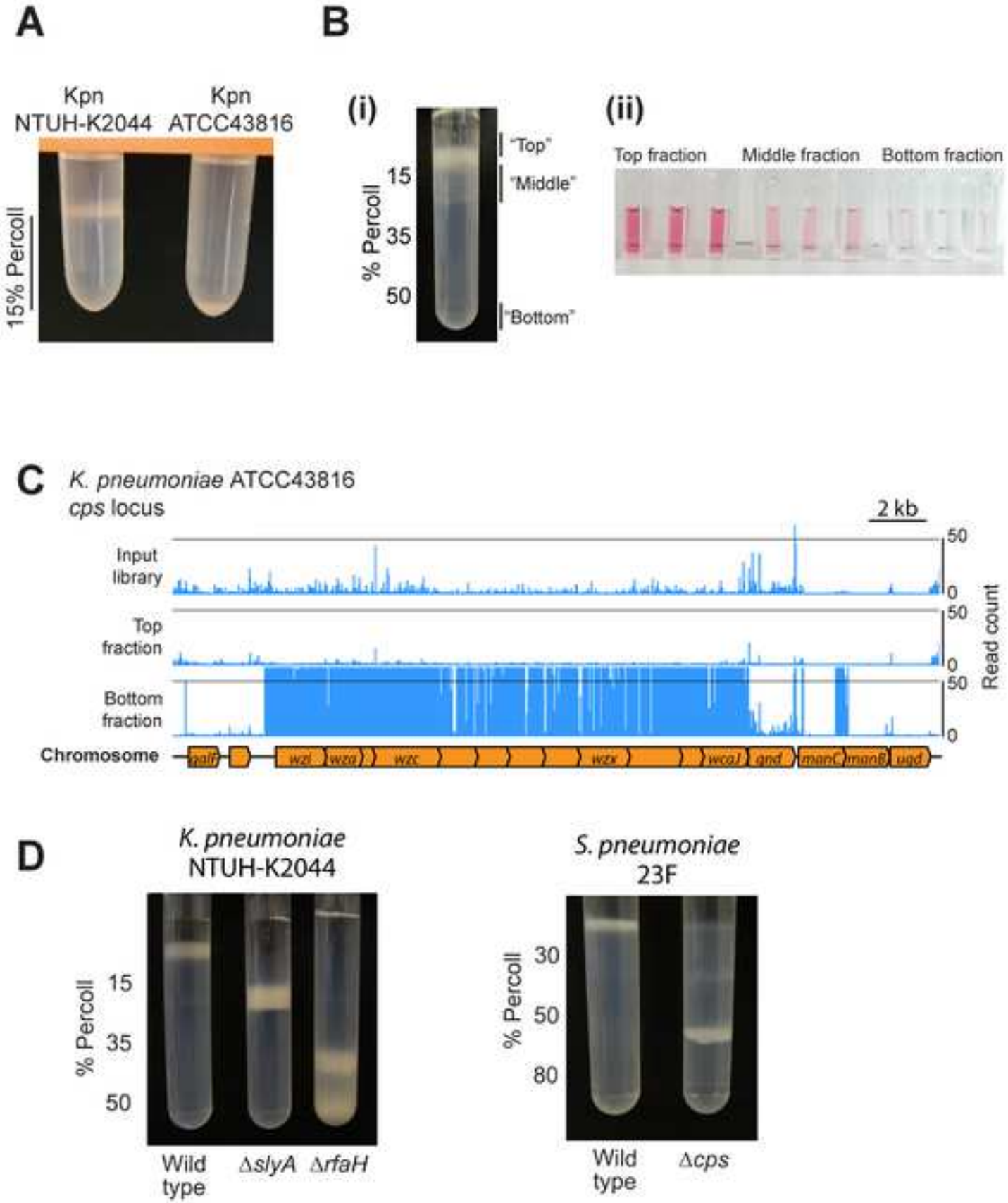


Figure



Name of Material/ Equipment	Company	Catalog Number
Percoll	GE Healthcare	17-0891-01
Centrifuge 5810R with Rotor A-4-81 and 500ml buckets	Eppendorf	5810 718.007
Adapters for 15ml tubes	Eppendorf	5810 722.004
Fixed andgle rotor F-34-6-38	Eppendorf	5804 727.002
2.6 - 7ml tube adapter	Eppendorf	5804 739.000
Centrifuge 5424 including Rotor FA-45-24-11	Eppendorf	5424 000.460
2ml tubes	Eppendorf	0030 120.094
1.5ml tubes	Eppendorf	0030 120.086
5ml polypropylene round bottom tube	Falcon	352063
1ml disposable syringe Luer slip	Becton Dickinson	300013
AGANI Needle 21G Green x 1.5"	Terumo	AN 2138R1
P1000 pipette and tips		
P200 pipette and tips		

Comments/Description

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Author(s): Feltwell et al

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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.