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# Method for concentrating viable microorganisms for microbial load determination and eliminating uncertainty from matrix effects from urine and whole blood



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## ABSTRACT

The ability to assess and eliminate the matrix effect in bioanalytical methods is critical for reproducibility, but sample preparation procedures necessary to address the matrix effect for microbiological methods could be significantly different if viable pathogens are required for downstream microbiological response analysis. A pure bacterial culture remains essential for virulence, antibiotic susceptibility, and phenotypic response studies in order to facilitate the understanding and treatment of caused diseases. Bacterial culture involves the collection, inoculation, incubation, growth, and detection of viable organisms while avoiding contamination throughout the entire process. The goal of this method is to concentrate viable pathogens directly from clinical specimens such as whole blood and urine while removing most interfering matrix components through pelleting in an enriched media, which is designed to facilitate the growth of clinically relevant microorganisms. Nonselective culture media with no inhibitors is used to permit the growth of most of the microorganisms present in the clinical samples studied. Most of the species implicated in clinical infections are mesophilic bacterial species, so the pelleting procedure is conducted at medium temperatures of 37°C to facilitate optimal growth.

- Viable bacterial pelleting for phenotypic response analysis.
- Concentration of bacteria by centrifugation and matrix component removal for direct-from-specimen molecular analysis.
- Viable pathogen recovery directly from whole blood and urine.

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## ARTICLE INFO

**Method name:** Centrifugation pelleting for viable pathogens directly from clinical specimens

**Keywords:** Lysis-centrifugation for bacterial pelleting, Matrix interference removal, Centrifugal concentration of viable bacteria

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## Specifications table

Subject Area	Immunology and Microbiology
More specific subject area	Sample preparation
Method name	Centrifugation pelleting for viable pathogens directly from clinical specimens
Name and reference of original method	Chiu ML, Lawi W, Snyder ST, Wong PK, Liao JC, Gau V. Matrix Effects—A Challenge toward Automation of Molecular Analysis. <i>JALA: Journal of the Association for Laboratory Automation</i> . 2010;15(3):233-242. doi:10.1016/j.jala.2010.02.001
Resource availability	Gao J, Jeffries L, Mach KE, Craft DW, Thomas NJ, Gau V, et al. A multiplex electrochemical biosensor for bloodstream infection diagnosis. <i>SLAS Technol</i> . 2017; 22(4): 466-474. doi: 10.1177/2211068216651232.
	All resources including hardware, protocols and materials necessary to reproduce the method are described in Method details.

## Method details

## Method overview

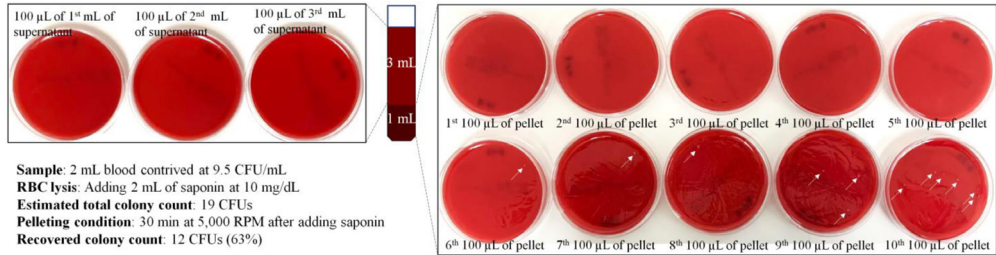
Centrifugation is a common laboratory practice used for harvesting bacteria from clinical specimens [1,2]. Centrifugation in essence involves compacting bacteria into a pellet with a wide range of gravitational forces (roughly ranging from 1,000 to 12,000  $\times g$ ), causing collisions against each other that result in shear forces on the bacterial cell surface, which may easily lead to entering stationary phase due to mechanical stress. Many experimental protocols choose high centrifugation speeds (revolutions per minute or RPM, gravitational force equivalent or  $g$ ) to increase the recovery rate while assuming that it does not affect the growth rate or viability. The protocol to obtain a highly concentrated viable bacteria pellet largely free of matrix constituents that can interfere with detection is specimen-type-specific [3]. Bacterial cells can be pelleted from clinical specimens such as urine and swab collection buffers without additional procedures if the viscosity and density are low. However, isolating bacteria from whole blood requires the selective lysis of red blood cells (RBCs) under conditions that do not disrupt target bacteria, repeated with a secondary size- and-density-based separation to concentrate bacteria from the resulting cell lysate for downstream microbiological response analysis. Chemical lysis can enable efficient and selective degradation of blood cells, but it could also adversely impact downstream assay performance. Serial dilution in culture medium is required to prevent ongoing chemical damage to target pathogens and supplement nutrients to support the controlled growth of viable pathogens. Undesirable dilution with culture medium can be compensated by repeatedly removing supernatant along with most interfering matrix components following sequential lysis and centrifugation steps. Due to the labor-intensive nature of these cumbersome steps, lab automation could be implemented to standardize the pelleting procedure to ensure reproducibility [4].

## Materials:

- Mueller-Hinton II (MH) broth, Cat. No. M5860, Teknova, Hollister, CA
- Saponin, Cat. No 47036, Sigma-Aldrich, St. Louis, MO
- BD Vacutainer Plus C&S tubes for urine pelleting, BD364954, VWR, Radnor, PA
- BD Vacutainer Lithium Heparin tube for blood collection, BD367884, VWR, Radnor, PA
- BD Vacutainer Serum tubes for blood pelleting, BD 367812, VWR, Radnor, PA
- Bacteria strain *E. coli* CDC 69

## Saponin reagent preparation protocol

1. Dissolve 50 mg of saponin powder in 50 mL DEPC water (Millipore Sigma, Cat. No. 9601-OP). Invert until powder is completely dissolved.



**Fig. 1.** Microbial recovery after the 1<sup>st</sup> cycle of saponin pelleting from 2 mL of negative blood contrived at 9.5 CFU/mL.

### Urine Pelleting Protocol

1. Centrifuge the 4-mL urine sample at 5000 revolutions per minute (RPM) or relative centrifugal force (RCF) of 4193 x g for 5 minutes.
2. Remove 3.5 mL of supernatant, leaving 0.5 mL of sample in the tube.

### Blood Pelleting Protocol

1. Add 2 mL of 10 mg/dL saponin to the 2mL blood sample.
2. Centrifuge at 5000 RPM for 15 minutes and remove 3 mL of supernatant, leaving 1 mL of sample.
3. Add 2 mL of 10 mg/dL saponin.
4. Centrifuge at 5000 RPM for 15 minutes and remove 2 mL of supernatant, leaving 1 mL of sample in the tube.

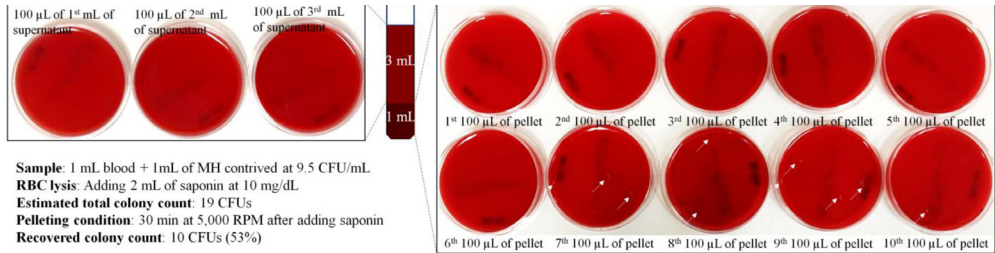
### **Method validation**

We wanted to verify that the described methods allowed for removal of matrix interference components and isolation of bacteria in urine and whole blood specimens. We contrived negative urine and whole blood specimens with clinically relevant levels of *E. coli* CDC 69, and performed the pelleting protocols described above. All specimens were obtained anonymously from remnant samples and were de-identified prior to testing under the approved NYP/Queens Institutional Review Board and joint master agreement.

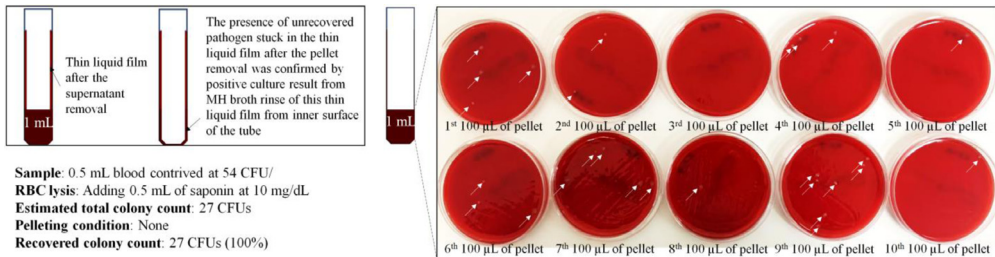
The microbial recovery rate is more critical for blood pelleting, since the range of colony count is a lot lower than that of urine. Therefore, we validated the microbial recovery rate for blood pelleting first. During the initial validation, we first investigated the microbial recovery after each cycle of saponin pelleting. Due to the high viscosity of whole blood and the abundance of red blood cells, the addition of saponin can help to promote mobility of target pathogens to be recovered in the pellet by reducing the viscosity and the number of red blood cells. At least two cycles of saponin pelleting are required to effectively remove the bulk of the blood matrix and recover bloodborne pathogens. To check for 100% microbial recovery rate, the colony count after each cycle of saponin pelleting was tabulated through colony counts of 100- $\mu$ L aliquots of the supernatant and pellet plated on blood agar. Initial assessment of microbial recovery with just one cycle of saponin pelleting was 63% as shown in Fig. 1.

The hypothesis is that the high viscosity of the blood mixture impacted either the microbial mobility or the retrieval of microorganisms from the inner surface inside the blood collection tube after centrifugation. To verify this hypothesis, 1mL of negative blood was diluted down with 1mL of MH broth, then contrived at 9.5 CFU/mL, and the recovery rate was 53% as shown in Fig. 2, indicating the reduced viscosity did not improve the recovery rate.

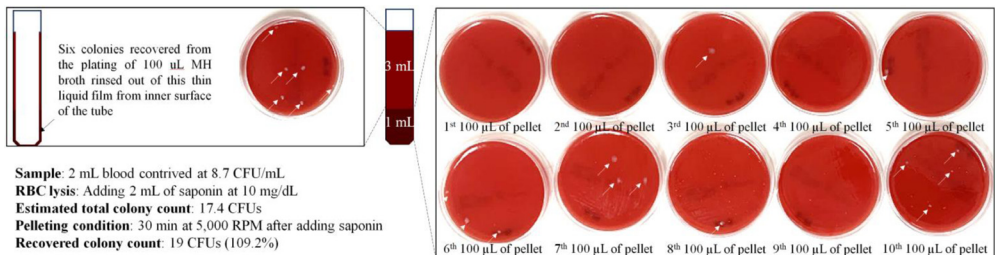
To further investigate the hypothesis that the unrecovered microbial colonies had adhered to the inner wall of the collection tube as a result of centrifugal force as shown in the left box in Fig. 3, a whole blood sample seeded with 54 CFU/mL was prepared and plated in saponin without pelleting, and 100% recovery was achieved due to all microorganisms being suspended in the bulk of the blood mixture as shown in Fig. 3.



**Fig. 2.** Microbial recovery after the 1<sup>st</sup> cycle of saponin pelleting from 1 mL of negative blood diluted with 1 mL of MH then contrived at 9.5 CFU/mL to reduce the viscosity, resulting in similar suboptimal recovery rate of 53%.



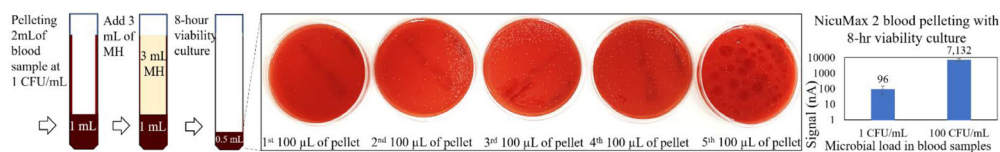
**Fig. 3.** Microbial recovery after addition of 0.5 mL saponin without pelleting from 0.5 mL of blood contrived at 54 CFU/mL without centrifugation to keep microbials suspended in the bulk of blood mixture. A recovery rate of 100% confirmed the hypothesis as shown in the left box.



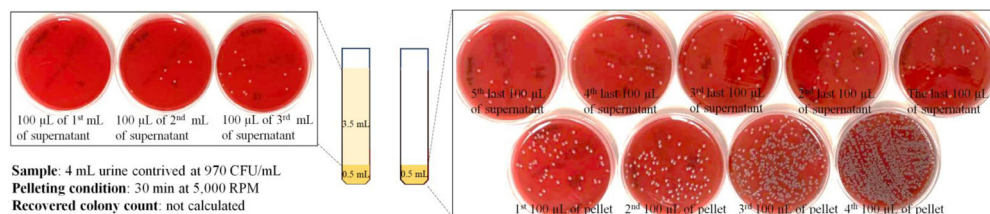
**Fig. 4.** Microbial recovery after the 1<sup>st</sup> cycle of saponin pelleting from 2 mL of blood contrived at 8.7 CFU/mL. The inner tube surfaced was rinsed with 100  $\mu$ L of MH and plated as shown in the left box to recover six colonies.

Lastly, we rinsed out the residual blood mixtures on the inner surface of the collection tube after removal of 3 mL of supernatant and 1 mL of pellet volume with 100  $\mu$ L of MH broth. Six colonies were recovered from the rinse out of the inner surface as shown in the left box in Fig. 4, and 13 colonies were recovered from the pellet indicating the viscosity and centrifugal force need to be carefully calculated to avoid losing colonies to the volume coating the inner surface of the collection tube.

After confirming that a recovery rate of 100% can be achieved by retrieval of remaining colonies stuck to the inner surface of the blood collection tube with MH broth, an additional viability culture including the addition of 3 mL of MH broth into the 1 mL blood pellet was performed to release all viable colonies. To ensure there would be at least one colony to be recovered, 2 mL of whole blood contrived at 1 CFU/mL was expected to contain 2 colonies following the protocol described above. The addition of 3 mL of MH and a viability culture allowed all colonies trapped in the cellular debris caused by the centrifugal force to be released and multiplied to a microbial load above the



**Fig. 5.** Microbial recovery from 2 mL of blood contrived at 1 CFU/mL followed by an 8-h viability culture to increase the microbial load above the limit of detection of the electrochemical-based molecular quantification assay of 16S rRNA content.



**Fig. 6.** Microbial recovery from 4 mL of urine sample contrived at 970 CFU/mL and the density check of the 500- $\mu$ L pellet

limit of detection of the biosensor to be used for downstream molecular analysis. After the viability culture, a high microbial load was expected, and the entire 4 mL of blood pellet mixture was pelleted down to 0.5 mL for quantification. As shown in Fig. 5, two sets of final pellets with 500  $\mu$ L each were quantified by both colony counts and electrochemical molecular quantification of the 16S rRNA content. The resulting colonies were too many to count, and the electrochemical signal level (in nano ampere or nA) was 96 nA from a 1 CFU/mL blood sample, which is higher than the limit of blank of 50 nA. The signal level from the blood sample contrived at 100 CFU/mL gave over 7,000 nA, which is close to the saturation of the reporting range of the electrochemical detection method.

A positive urine culture is often defined as the isolation of one or two uropathogens in pure culture at a growth level of  $\geq 10^3$  CFU/ml, and a negative urine culture is defined as no growth at  $\leq 10^3$  CFU/ml or growth of urogenital/skin contaminants only [5]. Therefore, a 100% recovery rate is not necessary for urinary microbial recovery. The urine pelleting protocol aims to remove the bulk of urine matrix components and concentrate the majority of uropathogens in the urine pellet. The viscosity of urine specimens is normally close to that of MH broth; therefore, saponin is not needed for urine pelleting. To investigate the recovery efficiency, a 4-mL urine sample seeded with 970 CFU/mL was spun down and a density check of 3.5 mL of supernatant and 0.5 mL of urine pellet was performed, as shown in Fig. 6, indicating that sufficient uropathogens were recovered for downstream molecular analysis.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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