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Title

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Permalink

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Journal

SLAS TECHNOLOGY, 25(1)

ISSN

2472-6303

Authors

Bradbury, Daniel W
Kita, Ashley E
Hirota, Kensuke
[et al.](#)

Publication Date

2020-02-01

DOI

10.1177/2472630319877377

Peer reviewed



Published in final edited form as:

SLAS Technol. 2020 February ; 25(1): 67–74. doi:10.1177/2472630319877377.

Rapid Diagnostic Test Kit for Point-of-Care Cerebrospinal Fluid Leak Detection

Daniel W. Bradbury^{1,*}, Ashley E. Kita^{2,*}, Kensuke Hirota¹, Maie A. St. John^{1,2,3,4}, Daniel T. Kamei¹

¹Department of Bioengineering, Henry Samueli School of Engineering and Applied Sciences, University of California Los Angeles, Los Angeles, California, USA

²Department of Head and Neck Surgery, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California

³Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California

⁴UCLA Head and Neck Cancer Program, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California

Abstract

Cerebrospinal fluid (CSF) leaks can occur when there is a communication between the intracranial cavities and the external environment. They are a common and serious complication of numerous procedures in otolaryngology, and if not treated, persistent leaks can increase a patient's risk of developing life-threatening complications such as meningitis. As it is not uncommon for patients to exhibit increased secretions post operatively, distinguishing normal secretions from those containing CSF can be difficult. Currently, there are no proven, available tests that allow a medical provider concerned about a CSF leak to inexpensively, rapidly, and non-invasively rule out the presence of a leak. The gold standard laboratory-based test requires that a sample is sent to a tertiary site for analysis where days to weeks may pass before results return. To address this, our group recently developed a semi-quantitative, barcode-style lateral-flow immunoassay (LFA) for the quantification of the beta-trace protein, which has been reported as an indicator of the presence of CSF leaks. In the work presented here, we created a rapid diagnostic test kit comprised of our LFA, a collection swab, dilution buffers, disposable pipettes, and instructions. Validation studies demonstrated excellent predictive capabilities of this kit in distinguishing between clinical specimens containing CSF and those that did not. Our diagnostic kit for CSF leak detection can be operated by an untrained user, does not require any external equipment, and can be performed in approximately 20 min, making it well-suited for use at the point-of-care. This kit has the potential to transform patient outcomes.

Corresponding Author: Daniel T. Kamei, Department of Bioengineering, University of California, 5121 Engineering V, 420 Westwood Plaza, Los Angeles, CA 90095-1600, USA. kamei@seas.ucla.edu.

*Designates co-first authorship

Declaration of Conflicting Interests

D.W. Bradbury, A.E. Kita, M.A. St. John, and D.T. Kamei are inventors of the device being studied and may benefit financially if the patent application is approved and the device is eventually manufactured and marketed.

Keywords

Cerebrospinal fluid leak; lateral-flow immunoassay; point-of-care; beta-trace protein; rapid diagnostic test

Introduction

Cerebrospinal fluid (CSF) leaks occur when there is a defect in the dura mater which results in a communication between the intracranial cavities and the external environment. CSF leaks are a common complication of numerous procedures in otolaryngology where the risk in endoscopic skull base surgery has been estimated at 13.8%, endoscopic sinus surgery at 0.17%, and cochlear implantation at 0.4%.¹⁻³ In fact, almost all procedures involving the sinuses, skull base, or ear carry some risk of a leak. Individuals who have not undergone surgical procedures are also at risk for CSF leaks. Approximately 80% of all leaks occur as a result of nonsurgical traumatic injuries, such as facial and skull bone fractures.⁴ While rare, some individuals even present with spontaneous CSF leaks such as CSF otorrhea from an encephalocele.⁵ Persistent CSF leaks can result in chronic headaches and discomfort, as well as increase a patient's risk of developing life-threatening complications such as bacterial meningitis and brain abscess.^{6,7} Therefore, early diagnosis and subsequent repair of CSF leaks is imperative to prevent these complications.

In the acute setting, assessment is performed using imaging modalities such as magnetic resonance imaging or computed tomography.⁸ If there is high enough clinical suspicion, such as obvious facial or skeletal deformities, or copious clear rhinorrhea or otorrhea, patients may be taken directly to the operating room for management. This involves first identifying the site of the leak and then using either native tissue or biocompatible materials to patch the affected site.

In an outpatient or postoperative hospital setting, diagnosing a CSF leak can be much more difficult. It is not uncommon for patients to exhibit heightened secretions after surgery and thus distinguishing normal secretions from those containing CSF can be difficult. Physicians could turn toward the imaging techniques used in the acute hospital settings; however, the cost is often difficult to justify for patients who otherwise seem healthy. Laboratory-based techniques for the detection of CSF may also be implemented. The laboratory gold standard technique involves the identification of the CSF-specific protein beta-2 transferrin using immunofixation electrophoresis.⁹⁻¹³ While proven to have excellent sensitivity and specificity in detecting CSF leaks, electrophoretic assays tend to be time-consuming, expensive, and labor-intensive. Additionally, these assays may not be readily available in some clinical laboratories, and thus, a sample must be sent to a tertiary site for analysis where days to weeks may pass before results return.¹⁴

As an alternative, researchers have investigated beta-trace protein (β TP), also known as lipocalin-type prostaglandin D2-synthase, as an indicator of CSF leaks. β TP is a ubiquitous protein present at various concentrations throughout the body, but is notably 1 to 2 orders of magnitude more concentrated in CSF than in serum and nasal secretions.¹⁵ As a result, numerous studies have demonstrated that nasal secretions with β TP concentrations

(measured using laser nephelometry) above a certain threshold are highly likely to be contaminated with CSF.^{16–20} A recent study by Bernasconi *et al.* demonstrated that concentrations of β TTP in nasal secretions ≥ 1.3 mg/L indicate the presence of a CSF leak, whereas concentrations of β TTP < 0.7 mg/L indicate the absence of a leak. β TTP concentrations between 0.7 and 1.29 mg/L (denoted the “gray zone”) required further analysis and comparison with the patient’s β TTP serum level to confirm the presence or absence of a CSF leak. Overall, this approach showed a sensitivity and specificity of 98.3% and 96%, respectively.²¹ While the nephelometric assay is more rapid than electrophoresis, it still requires centralized laboratory equipment that may not be available in every clinical setting. In fact, there are still no proven, widely available diagnostic techniques that allow physicians to rule out the presence of a CSF leak in a rapid, affordable, and non-invasive manner.

To address this, our lab has previously developed a semi-quantitative, barcode-style lateral-flow immunoassay (LFA) for the detection of β TTP.²² Using this LFA, we demonstrated the ability to identify serum samples spiked with CSF from those that were not. While these results were promising, we had yet to validate our LFA using samples from patients with or without CSF leaks. Additionally, prior to running these assays, a sample dilution step was performed using precision laboratory pipettes which may not be readily available in all clinical settings. In the work presented here, we have developed a rapid diagnostic test kit containing our semi-quantitative LFA for detecting β TTP, a collection swab, diluent buffers, and disposable quantitative transfer pipettes. The kit we have developed (described herein) is simple to use, does not require prior user training, or additional supplies (such as laboratory pipettes), which make it facile and suitable for use at the point of care. We validated our kit by testing a variety of different samples collected from regions surrounding the brain and demonstrated its ability to serve as a rapid, low-cost screening tool for the detection of CSF leaks. To our knowledge, this is the first rapid diagnostic test kit developed for the detection of CSF leaks.

Materials and Methods

Formation of anti- β TTP antibody-conjugated gold nanoprobles (GNPs)

All reagents and materials were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Gold nanoprobles for the detection of β TTP were prepared according to our previously reported protocol.²² Briefly, anti- β TTP gold nanoprobles (GNPs) were formed by first mixing 35 μ L of a 0.1 M sodium borate solution (pH 9) with a 1 mL suspension of 40 nm citrate-capped gold nanoparticles (Nanocomposix, San Diego, California). Subsequently, 8 μ g of anti- β TTP detection antibodies were added and incubated for 30 min at room temperature to allow the formation of dative bonds between the antibodies and the surface of the gold nanoparticles. The surface of the gold nanoparticles was then passivated to prevent the nonspecific binding of other proteins by adding 100 μ L of a 10% w/v bovine serum albumin (BSA) solution and allowing the mixture to react for 10 min. Lastly, free antibodies were removed from the suspension by 3 centrifugation steps at 9000 rcf for 6 min, followed by a final resuspension in 100 μ L of a 0.1 M sodium borate buffer (pH 9).

Preparation of barcode-style LFA test strips

LFA test strips were constructed by overlapping and securing different functional paper pads on an adhesive backing. Each LFA test strip included a sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad. Each sample pad was made by dehydrating 12 μL of 1% w/v BSA in 0.1 M Tris buffer (pH 9) onto a 3×10 mm fiber glass paper pad. Each conjugate pad was made by dehydrating a 12 μL solution containing 2.5 μL GNPs and 1% w/v BSA overall onto a 3×10 mm fiber glass paper pad. Both the sample pads and conjugate pads were dehydrated under very low pressure using a Labconco FreeZone 4.5 lyophilizer (Fisher Scientific, Hampton, New Hampshire) for 2 hours.

The nitrocellulose membrane was prepared by immobilizing antibodies at three different test line locations (T1, T2, and T3) and a control line location. The concentrations of the anti- βTP detection antibodies immobilized at T1, T2, and T3, were 2, 0.5, and 0.35 mg/mL, respectively. The control line was composed of an anti-mouse IgG secondary antibody immobilized at 0.5 mg/mL. The antibody immobilization process was performed by printing solutions of antibodies in 25% w/v sucrose onto a nitrocellulose membrane using an Automated Lateral Flow Reagent Dispenser (Claremont BioSolutions, Upland, California) and a Fusion 200 syringe pump (Chemyx, Stafford, Texas) at a flow rate of 250 $\mu\text{L}/\text{min}$. The printed membrane was then left in a vacuum-sealed desiccant chamber overnight. After antibody immobilization, the nitrocellulose membrane was blocked by immersing it in a solution of 1% w/v BSA in 0.1 M Tris buffer for 1 h followed by dehydration overnight at low pressure with a lyophilizer.

To assemble the LFA test strip, the nitrocellulose membrane was first mounted onto the adhesive backing. The absorbent pad was adhered onto the adhesive backing just downstream of the control line, where it overlapped the nitrocellulose membrane by 2 mm. Next, the conjugate pad was adhered onto the backing just upstream of the test lines, where it overlapped the nitrocellulose membrane by 1 mm. Lastly, the sample pad was adhered to the region of adhesive backing upstream of the conjugate pad while also overlapping the conjugate pad by 2 mm.

Preparation of diagnostic kit for CSF leak detection

A rapid diagnostic test kit was assembled containing one barcode-style LFA test strip, one Flexible Minitip Flocked Swab (Becton, Dickinson and Company, Franklin Heights, NJ), two disposable Samco Exact Volume Transfer pipettes (Thermo Scientific, Waltham, MA) with a capacity of 100 μL , diluent buffers, and user instructions. Tube A contained 2,320 μL of phosphate-buffered saline (PBS). Tube B contained 450 μL of PBS mixed with 450 μL of running buffer (0.4% BSA, 0.6% Tween 20, 0.2% polyethylene glycol, 0.1M Tris buffer pH 9). Tube C was supplied empty. All components were placed in a resealable bag with desiccant for storage.

Operation and validation of rapid diagnostic test kit for CSF leak detection

The schematic representation of the user steps for our developed diagnostic test kit is shown in Fig. 1. To determine whether a sample fluid contained CSF, the fluid was first absorbed into the collection swab. The swab was then inserted into the buffer in tube A and twirled

around the edge of the tube for 10 s at 2 rotations per second in order to release the sample from the swab into the buffer. Tube A was then inverted 5 times to ensure the released sample was well-mixed. Next, 100 μ L of the liquid in tube A was transferred to tube B using one of the provided disposable transfer pipettes. Tube B was then inverted 5 times to ensure complete mixing. The release of the sample from the swab into tube A and the subsequent transfer into tube B resulted in an overall 300-fold dilution of the original sample. Lastly, 100 μ L of the solution in tube B was transferred into tube C where the barcode-style LFA test strip was also inserted. After 20 min, the results were interpreted where the formation of 0 and 1, 2, or 3 visible test lines indicated a diagnosis of negative, gray zone, or positive, respectively. Photographs of the test strips were taken by a Nikon D3400 digital camera (Nikon, Tokyo, Japan) in a controlled lighting environment.

We first evaluated the ability of our diagnostic test kit to detect for β TTP by testing samples containing purified, recombinant β TTP in PBS. Then we evaluated a variety of different patient samples collected at the Ronald Reagan UCLA Medical center which were either negative or positive for the presence of CSF. These included fluid collected from skull base sites during endoscopic transnasal transsphenoidal (TNTS) surgeries, superior semicircular canal dehiscence (SSCD) repair surgeries, and brain tumor resection surgeries. Rhinorrhea and otorrhea specimens from healthy volunteers and patients with suspected CSF leaks were also included (University of California Los Angeles [UCLA] Institutional Review Board approval 17-001065).

Results

Barcode-style LFA principle and interpretation

Similar to our previous work, the barcode-style LFA used in our kit was designed such that the presence of the target biomarker, β TTP, would produce 1 to 3 visible test lines, because the β TTP would become sandwiched between the GNPs and the test line antibodies. Each test line has a cutoff value, which is the minimum concentration of β TTP required for that line to become visible to the user. In designing a barcode-style LFA, the cutoff value for each line could be adjusted by varying the concentrations of the immobilized antibodies. Regardless of the presence of β TTP, the anti-mouse IgG secondary antibodies immobilized at the control line would bind to the primary antibodies on the GNPs, capturing them. This would result in the formation of a visible red control line to indicate successful flow of the sample solution through the LFA test strip and thus valid test results.

The cutoff values for each test line of our barcode-style LFA were adjusted to correspond with the threshold values proposed by Bernasconi *et al.*²¹ β TTP concentrations <0.7 mg/L would result in the appearance of 0 or 1 test line and indicate no CSF leak. Between 0.7 and 1.3 mg/L of β TTP, 2 test lines would appear and classify the sample as in the “gray zone.” Samples that fall within the gray zone cannot be confidently diagnosed as negative or positive for the presence of CSF and therefore should undergo further testing before a clinical decision is made. Lastly, samples with β TTP concentrations >1.3 mg/L would result in the appearance of 3 visible test lines, indicating the presence of a CSF leak.

Detection of purified β TP

The performance of our rapid diagnostics test kit for the detection of CSF leaks was first evaluated by testing samples of purified, recombinant β TP in PBS. Solutions containing different concentrations of β TP were collected on a swab, transferred into diluent solutions, and then applied to the barcode-style LFA test strip. For all experiments, pink control lines became visible on the LFA test strips indicating valid test results. β TP was also able to be detected at all concentrations tested, indicated by the formation of at least one pink test line on each LFA strip. More specifically, when the concentration of β TP was 0.3 mg/mL, a visible test line appeared at the T1 location. At a β TP concentration of 0.9 mg/mL, test lines appeared at the T1 and T2 locations. Lastly, at 3 mg/mL of β TP, test lines appeared at the T1, T2, and T3 locations (Fig. 2).

Detection of clinical samples

Next we evaluated the performance of our diagnostic test kit with clinical samples. To do this, we tested 22 samples from 21 patients. Of these samples, 8 contained CSF from the periphery of the brain and 14 did not contain CSF. The demographics of the study population as well as the sample origins are summarized in Table 1. 13 samples were collected from the site of surgical procedures, 8 were rhinorrhea specimens, and 1 was an otorrhea specimen.

Each clinical specimen was collected on a swab, transferred into diluent solutions, and applied to the barcode-style LFA test strip. The results that were obtained are summarized in Table 2 (photographs of the test strips are provided in the electronic supplementary information). Of the 4 surgical specimens containing CSF, 3 produced true positive results, and 1 produced a gray zone result. On the other hand, of the 9 surgical specimens that did not contain CSF, 7 produced true negative results, and 2 produced gray zone results. Rhinorrhea specimens were also examined, where all 3 specimens containing CSF resulted in true positive results, and all 5 specimens that did not contain CSF resulted in true negative results. An otorrhea specimen containing CSF was also tested and it produced a true positive result.

Three different strategies for interpreting the diagnostic test kit results were evaluated. In the first method, the gray zone results were interpreted as negative. For the second strategy, the gray zone results were interpreted as being positive. Lastly, in the third strategy, gray zone results were interpreted as being equivocal and ambiguous. The first two interpretation strategies allowed us to simplify the obtained results to provide the user with a binary answer as to whether the tested sample does or does not contain CSF. Ultimately this allowed us to collapse the obtained results onto a 2x2 contingency table to characterize the diagnostic test kit performance (Table 3). In the first method, where the gray zone was interpreted as a negative result, we obtained a sensitivity of 87.5%, specificity of 100%, positive predictive value (PPV) of 100%, negative predictive value (NPV) of 93.3%, positive likelihood ratio (+LR) of ∞ , and negative likelihood ratio (-LR) of 0.125. In the second method, where the gray zone was interpreted as positive, we obtained a sensitivity of 100%, specificity of 85.7%, PPV of 80%, NPV of 100%, +LR of 7, and -LR of 0. In the third interpretation method, where gray zone results are considered equivocal and ambiguous, the

results were not binary so they could not be analyzed using a 2×2 contingency table and thus sensitivity, specificity, PPV, and NPV could not be calculated. However, the likelihood ratio (LR) for each detection region could be calculated, where a negative result (0 or 1 test line) had an LR of 0, a gray zone result had an LR of 0.88, and a positive result had an LR of ∞ (Table 4).

Discussion

The early and accurate diagnosis of CSF leaks is vital for the prevention of life-threatening complications such as meningitis. The quantification of β TTP in rhinorrhea and otorrhea specimens using nephelometry has previously been reported as an effective method of diagnosing CSF leaks. While it is more rapid than the gold standard laboratory technique of beta-2 transferrin electrophoresis, the nephelometry assay does require the use of expensive, centralized laboratory equipment that is not widely available in all clinical settings. In this work, we created a rapid diagnostic test kit that utilized a barcode-style LFA, and validated its use for the equipment-free collection, dilution, and detection of CSF.

We first tested the rapid diagnostic test kit with purified, recombinant β TTP in PBS. Our results demonstrate that the diagnostic test kit can detect for β TTP in a semi-quantitative manner, as seen by the increase in the number of red test lines visible on the LFA test strip with an increase in the concentration of β TTP. More specifically, positive results were observed at 3 mg/L, a gray zone result was observed at 0.9 mg/L of β TTP, and a negative result was observed at 0.3 mg/L of β TTP. These results correspond relatively well with the cutoff values proposed by Bernasconi *et al.*²¹ Additionally, these test results obtained with swabs and disposable transfer pipettes were comparable to that of our previous work which used no swabs and high quality laboratory pipettes for collection and dilution. This is significant because it demonstrates that the samples could be collected, released, and diluted in a quantitative manner using low cost and disposable materials.

All clinical specimens produced true negative and true positive test results, with the exception of three surgical specimens that produced results in the gray zone. One of these specimens contained CSF while the other two did not. All three were collected at the sites of endoscopic surgeries, where a significant amount of blood was collected on the swab. The mean concentration of β TTP in human serum has previously been reported as 0.59 mg/L with a range of 0.38 – 0.86 mg/L.¹⁷ Recall that our diagnostic test kit produced gray zone results (two test lines) at 0.9 mg/L of recombinant β TTP. Thus, it is possible that the gray zone results obtained for the two surgical specimens without CSF is a result of contamination with blood that has a β TTP concentration at the upper end of the reported range. It is worth noting that the other seven surgical specimens with true negative detection also contained blood; however, the blood could have been more dilute or at the lower end of the reported β TTP concentration range for serum. Regarding the surgical specimen containing CSF which produced gray zone results, it is likely that the CSF was significantly diluted in other secretions to below the positive detection threshold of our diagnostic kit.

We evaluated three different strategies for interpreting and analyzing our diagnostic test kit results. The first two methods involved simplifying our results of negative, gray zone, or

positive to a binary readout of negative or positive. The performance of the first method, where gray zone results were interpreted as negative, is conducive for a rule-in test, because it was highly specific where false positive results are extremely unlikely, and thus a positive result could be used to confidently confirm the presence of the CSF leak. For the second method, where gray zone results are interpreted as positive, our diagnostic test kit became an effective rule-out test, because it has high sensitivity where false negatives are extremely unlikely and thus it can be used to confidently send healthy patients home.

In the case that we designed our diagnostic test kit to only provide binary results, such as if the LFA test strip was printed with one test line as opposed to three test lines, a rule-out test would be desired. From a clinical perspective, a rule-out test is advantageous because it would allow clinicians to more confidently send healthy patients home who were suspected of having a CSF leak and otherwise would have been scanned and admitted into the hospital for observation or further invasive procedures. This would ultimately save hospital resources and minimize the number of healthy patients who would undergo unnecessary and costly workups such as MRI or CT imaging. While the rule-out test would be the most useful binary test for the detection of CSF leaks, it is not without its disadvantages. Note that the rule-out test has a very high sensitivity (100%) at the cost of a slightly lower specificity (85.7%). The PPV was calculated as 80% meaning that 20% of the positive test results were actually false positives. Therefore, further testing would have to be performed for all patients with positive test results prior to intervention in order to confirm the presence of a leak.

To avoid having to choose between a binary test with either high sensitivity or high specificity, a non-binary third interpretation strategy is presented, where the gray zone results are treated as their own category indicating equivocal and ambiguous test results. In the case that the pre-test odds for some unhealthy condition are known, likelihood ratios (LRs) multiply the pre-test odds to determine the post-test odds which then can be converted to the post-test probability that a patient has some condition of interest.²³ Thus, the LR values of 0 for negative and ∞ for a positive test indicates that, regardless of the pre-test probability, a negative test result confirms that the patient does not have a CSF leak, and a positive test result confirms a CSF leak. This is an improvement over the previously discussed second strategy, where 20% of positive results were false positives. Lastly, gray zone results had an LR of 0.88, which provides very little useful information to influence the clinical diagnosis because it is close to 1. Therefore, patients who appear healthy but test within the gray zone would be identified as being at risk of having a CSF leak, and further non-invasive testing, such as beta-2 transferrin electrophoresis, should be performed prior to clinical decision making. It is worth noting that in our study, only 14% of patient samples produced gray zone results which would require further confirmatory testing. This is a significant improvement over the 45% of patients that would be required to receive further testing if the binary rule-out test format was implemented.

Altogether, the diagnostic test kit developed in this study costs under \$4 to produce at the laboratory scale and produces results in approximately 20 min. Using our kit, clinicians would be able to rapidly and accurately identify healthy individuals from a pool of patients with suspected CSF leaks. Ultimately, this would save hospital resources by minimizing the number of healthy patients who would otherwise be admitted to the hospital for multiday

observation while waiting for beta-2 transferrin electrophoresis results, as well as minimize the number of unnecessary and costly workups such as MRI or CT imaging.

Conclusions

In summary, we have developed the first rapid diagnostic test kit for the detection of CSF leaks. Every step from sample collection through results interpretation can be performed with minimal training and requires only the provided disposable materials which make this kit well suited for use as a point-of-care diagnostic. Validation studies from testing clinical samples that either did or did not contain CSF demonstrated excellent predictive capabilities. Overall our test kit has the potential to influence the clinical decision-making process by preventing unnecessary workups for healthy patients and rapidly confirming the presence of leaks in unhealthy patients. Ultimately this will lead to the improved management and treatment of CSF leaks. This kit has the potential to transform patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

The authors disclosed receipt of the following financial support for this research, authorship, and/or publication of this article: This work was supported by the National Institutes of Health grant 1R21 NS099800-01 and a 2016 AAO-HNS Core Resident Research Award.

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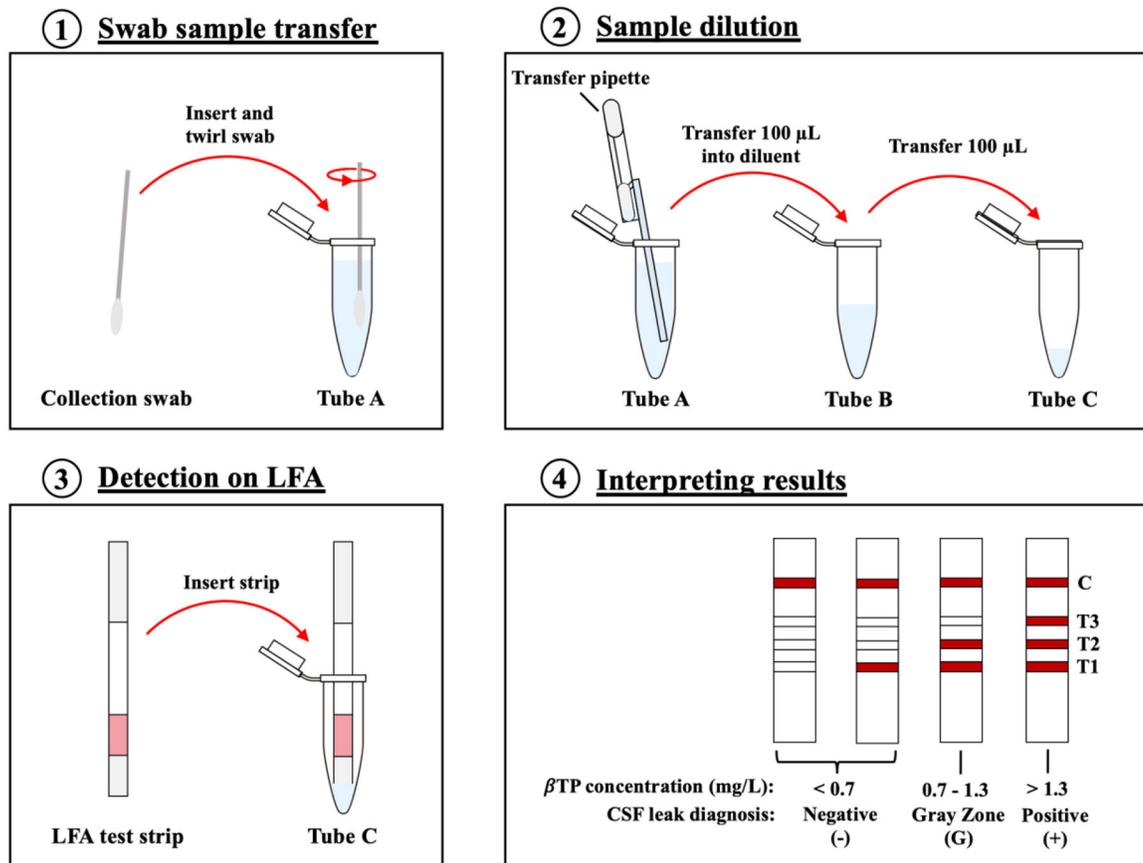


Figure 1:

Schematic representation of the procedure for operating our rapid diagnostic test kit for the detection of CSF leaks. (1) Swab with collected sample is dipped and twirled in tube A buffer to release sample. (2) Released sample from tube A is further diluted in tube B using disposable transfer pipettes and then transferred to tube C. (3) Barcode-style LFA test strip is inserted into the fluid in tube C for 20 min. (4) Test results are interpreted by the user.

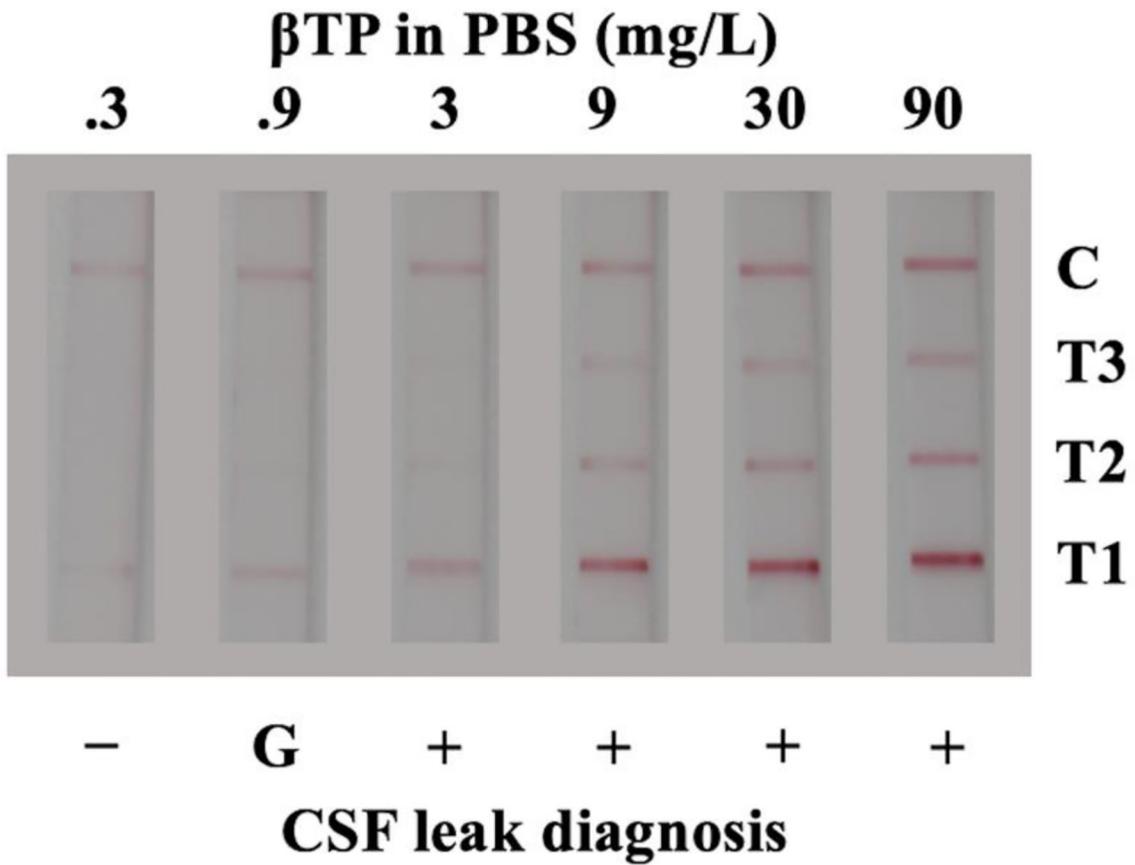


Figure 2: Diagnostic test kit results for the detection of recombinant β TP in PBS. Negative (-), Gray Zone (G), and Positive (+) results are indicated.

Table 1:

Patient characteristics

Patient number (female/male)	21 (13/8)
Age, years (mean \pm SD)	45 \pm 14
Age range, years	18–70
Sample number	22
Surgical specimen, n	13
Rhinorrhea specimen, n	8
Otorrhea specimen, n	1

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Table 2:

Summarized diagnostic test kit results

Clinical samples	Diagnostic test kit results			Total
	Negative	Gray	Positive	
Surgical specimen with CSF	0	1	3	4
Surgical specimen without CSF	7	2	0	9
Rhinorrhea specimen with CSF	0	0	3	3
Rhinorrhea specimen without CSF	5	0	0	5
Otorrhea specimen with CSF	0	0	1	1
Total	12	3	7	22

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Table 3:

Diagnostic performance using binary assay interpretation

Interpretation	Sensitivity	Specificity	PPV	NPV	+LR	-LR
Gray zone as negative	87.5%	100%	100%	93.3%	∞	0.125
Gray zone as positive	100%	85.7%	80%	100%	7	0

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Table 4:

Diagnostic performance using non-binary assay interpretation

Assay result	n (%) with CSF	n (%) without CSF	% with CSF/% without CSF	Likelihood ratio
Negative	0 (0%)	12 (85.7%)	0/85.7	0
Gray zone	1 (12.5%)	2 (14.3%)	12.5/14.3	0.88
Positive	7 (87.5%)	0 (0%)	87.5/0	∞
Total	8 (100%)	14 (100%)	-	-

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