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Title: Handheld Device for Saliva Pretreatment to Improve Cortisol Detection

Authors and Affiliations:

Kang Hyeon Kim^{1,*}, Na Eun Lee^{1,2,*}, Ho Seung Song³, Yong Kyoung Yoo⁴, Dohwan Lee¹

¹Department of Electrical Engineering, Kwangwoon University

²School of Biomedical Engineering, Korea University

³Department of Electronic Engineering, Catholic Kwandong University

⁴Department of Electronic and Semiconductor Engineering, Kangwon National University

*These authors contributed equally to this work

Corresponding Authors:

Ho Seung Song
Yong Kyoung Yoo
Dohwan Lee

hssong@cku.ac.kr
ykyoo@kangwon.ac.kr
dlee@kw.ac.kr

Email Addresses for All Authors:

Kang Hyeon Kim
Na Eun Lee
Ho Seung Song
Yong Kyoung Yoo
Dohwan Lee

kgh4436@kw.ac.kr
naeun5582@korea.ac.kr
hssong@cku.ac.kr
ykyoo@kangwon.ac.kr
dlee@kw.ac.kr

Author Questionnaire

1. **Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**
3. **Filming location:** Will the filming need to take place in multiple locations? **No**
4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 09

Number of Shots: 26

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Kang Hyeon Kim:** Our research focuses on saliva pretreatment to improve the reliability of cortisol detection.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Kang Hyeon Kim:** This protocol can be applied to saliva-based biomarker analysis using the lateral flow assay.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Kang Hyeon Kim:** This protocol allows researchers to evaluate salivary cortisol after removing proteins and mucins.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Kang Hyeon Kim:** After filtration, samples can be analyzed by SDS-PAGE, protein assays, ELISA, or LFA.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Kang Hyeon Kim:** Future studies can validate this platform using clinical saliva samples with different cortisol concentrations.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Saliva Sample Preparation and SaliFilter Device Fabrication Using RBCM-coated Filter Membrane

Demonstrator: Kang Hyeon Kim

- 2.1. To begin, gently mix the commercially obtained de-identified pooled human saliva sample before use to ensure homogeneity [1]. Store the saliva samples at 4 degrees Celsius and use them within the supplier's recommended storage period [2]. Thaw the pale pink, red blood cell membrane, or RBCM (R-B-C-M) pellet prepared and stored at minus 80 degrees Celsius, as per a standard protocol [3].
 - 2.1.1. WIDE: Talent gently mixing the saliva sample. *Videographer: Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent placing the saliva samples into storage at 4 °C.
 - 2.1.3. Talent thawing the RBCM pellet retrieved from – 80 °C
- 2.2. Prepare a polyethersulfone, or PES (P-E-S) membrane with a pore size of 30 nanometers and a diameter of 13 millimeters [1]. Deposit 100 microliters of 2 percent weight per volume RBCM solution onto one side of the PES membrane [2]. Gently spread or dispense the solution dropwise until the entire membrane surface is fully wetted [3].
 - 2.2.1. Talent preparing a PES membrane with a pore size of 30 nm and a diameter of 13 mm.
 - 2.2.2. Talent depositing RBCM solution onto one side of the PES membrane.
 - 2.2.3. Talent wetting the entire membrane surface with the RBCM solution.
- 2.3. Incubate the membrane at 50 degrees Celsius for 30 minutes to ensure uniform coating and drying [1]. Store the coated membrane at room temperature and use it within 15 days after preparation [2].
 - 2.3.1. Talent placing the membrane into the incubator.
 - 2.3.2. Talent storing the coated membrane at room temperature.
- 2.4. To fabricate the Salifilter device, place the RBCM-coated PES membrane into the designated filter holder [1]. Assemble the device by securely connecting the inlet and outlet components [2]. Attach a 1-milliliter syringe to the device inlet [3].
 - 2.4.1. Talent placing the RBCM-coated PES membrane into the designated filter holder.
 - 2.4.2. Talent connecting the inlet and outlet components of the device.

2.4.3. Talent attaching a 1 milliliter syringe to the device inlet.

3. Salivary Pretreatment and Cortisol Detection by Lateral Flow Assay

3.1. Load 500 microliters of saliva into the syringe connected to the SaliFilter device [1]. Apply gentle manual pressure to pass the saliva sample through the membrane for approximately 2 minutes [2]. Collect the pretreated saliva from the outlet [3] and use it immediately or store it at 4 degrees Celsius until further analysis [4].

3.1.1. Talent loading saliva into the syringe connected to the SaliFilter device.

3.1.2. Talent applying gentle manual pressure to pass the saliva sample through the membrane.

3.1.3. Talent collecting pretreated saliva from the outlet.

3.1.4. Talent placing the pretreated sample into storage at 4 °C.

3.2. After protein detection by SDS-PAGE, apply 150 microliters of pretreated saliva to the sample pad of the LFA (*L-F-A*) strip for cortisol detection using the lateral flow assay [1]. Allow the sample to migrate along the strip for 10 minutes [2]. Capture images of the test results using a smartphone or imaging system [3].

3.2.1. Talent applying pretreated saliva to the sample pad of the lateral flow assay strip.

3.2.2. Shot of the sample migrating along the strip.

3.2.3. Talent capturing images of the test results using a smartphone or imaging system.

3.3. Drag and drop the captured LFA file into the ImageJ software window [1]. Click on **Image**, choose **Color**, and then select **Split channels** to separate the red, green, and blue channels [2].

3.3.1. SCREEN: 79158_screenshot_1. 00:00 – 00:05

3.3.2. SCREEN: 79158_screenshot_1. 00:09 – 00:24. *Video Editor: Please emphasize the arrow clicking on Image option on the left side of the screen at 00:09 timestamp and emphasize the words “red”, “blue” and “green” in the three image panels at timestamp 00:22.*

3.4. Close the red and blue channels and use the green channel image for test-line intensity analysis [1]. Use the rectangle tool to select the region of interest, or ROI (*R-O-I*) of 100 by 10 pixels covering the test line [2]. Open the other LFA strip images and apply the same ROI size across all samples [3].

3.4.1. SCREEN: 79158_screenshot_2. 00:00 – 00:11. *Video Editor: Please emphasize the word “green” on top of the image panel at timestamp 00:09*

- 3.4.2. SCREEN: 79158_screenshot_2. 00:12 – 00:32. *Video Editor: Please emphasize clicking on the rectangle tool in the top left corner of the Image J window.*
- 3.4.3. SCREEN: 79158_screenshot_2. 00:50 – 01:10. *Video Editor: Please emphasize the rectangle shape ROI placed on all 3 sample images at timestamp 01:09.*
- 3.5. Click on **Analyze** and choose **Measure** to obtain the mean intensity value of the test-line ROI [1]. Next, select an adjacent blank membrane region with the same ROI size and measure the background intensity [2]. Export the values to an Excel spreadsheet and calculate the normalized test-line intensity by subtracting the background intensity from the test-line intensity [3].
 - 3.5.1. SCREEN: 79158_screenshot_3. 00:00–00:10 *Video Editor: Please emphasize the arrow clicking on “Analyze” and highlight the value under “Mean” column of the Results window.*
 - 3.5.2. SCREEN: 79158_screenshot_3. 00:11–00:27
 - 3.5.3. SCREEN: 79158_screenshot_3. 00:33–00:40; 01:09–01:16

Results

4. Results

- 4.1. Evaluation of filtration capability of SaliFilter by comparing turbidity changes against commercial saliva collection kits showed high turbidity in untreated saliva [1], whereas SaliFilter-treated samples appeared visibly clear [2]. Samples processed using commercial kits showed only limited improvement in clarity [3].
 - 4.1.1. LAB MEDIA: Figure 2A. *Video editor: Please emphasize the 1st tube labelled "Whole Saliva"*
 - 4.1.2. LAB MEDIA: Figure 2A. *Video editor: Please emphasize the 4th tube labelled "Salifilter"*
 - 4.1.3. LAB MEDIA: Figure 2A. *Video editor: Please emphasize the 2nd and 3rd tubes labelled "Salivette" and "Supersal"*
- 4.2. Salivary protein quantification by absorbance at 280 nanometers showed an approximate 69% reduction following filtration [1]. A Bradford assay showed a marked decrease in color intensity in SaliFilter-treated samples corresponding to an approximately 80% reduction in total protein concentration [2].
 - 4.2.1. LAB MEDIA: Figure 2B. *Video editor: Please emphasize the value 68.802 % in the graph.*
 - 4.2.2. LAB MEDIA: Figure 2D. *Video editor: Please emphasize the value 79.45 % in the graph.*
- 4.3. Capillary flow analysis demonstrated that removing mucins restored flow performance in pretreated saliva [1]. SDS-PAGE analysis confirmed that mucins were retained upstream of the membrane [2] and were absent from the processed samples [3].
 - 4.3.1. LAB MEDIA: Figure 3A. *Video editor: Please emphasize the maroon bar along with its X-axis label.*
 - 4.3.2. LAB MEDIA: Figure 3B. *Video editor: Please emphasize the lane labelled "2" on the membrane image along with the figured legend "Lane 2: Retained sample".*
 - 4.3.3. LAB MEDIA: Figure 3B. *Video editor: Please emphasize the lane labelled "3" on the membrane image along with the figured legend "Lane 3: Processed sample".*

Pronunciation guides:

1. De-identified
Pronunciation link: <https://www.merriam-webster.com/dictionary/de-identified>
IPA: /ˌdiː.ərɪˈden.təˌfaɪd/
Phonetic Spelling: dee·eye·den·tuh·fyd
2. Homogeneity
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogeneity>
IPA: /ˌhɒʊ.mə.dʒəˈniː.ə.ti/
Phonetic Spelling: hoh·muh·juh·nee·uh·tee
3. Thaw
Pronunciation link: <https://www.merriam-webster.com/dictionary/thaw>
IPA: /θɔː/
Phonetic Spelling: thaw
4. Polyethersulfone
Pronunciation link: <https://www.howtopronounce.com/polyethersulfone>
IPA: /ˌpɑː.li.ɪ.θəˈsʌl.fʊn/
Phonetic Spelling: pah·lee·ee·ther·sul·fohn
5. Membrane
Pronunciation link: <https://www.merriam-webster.com/dictionary/membrane>
IPA: /ˈmem.breɪn/
Phonetic Spelling: mem·brayn
6. Nanometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/nanometer>
IPA: /ˈnæn.oo.miː.tə/
Phonetic Spelling: nan·oh·mee·ter
7. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /ˈmaɪ.kroʊ.liː.tə/
Phonetic Spelling: my·kroh·lee·ter
8. Deposit
Pronunciation link: <https://www.merriam-webster.com/dictionary/deposit>
IPA: /dɪˈpɑː.zɪt/ (verb)
Phonetic Spelling: dih·poz·it
9. Dispense
Pronunciation link: <https://www.merriam-webster.com/dictionary/dispense>
IPA: /dɪˈspens/
Phonetic Spelling: dih·spens
10. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /ˈɪŋ.kjəˌbeɪt/
Phonetic Spelling: ing·kyuh·bayt
11. Fabricate
Pronunciation link: <https://www.merriam-webster.com/dictionary/fabricate>

IPA: /'fæb.rɪ.keɪt/

Phonetic Spelling: fab·ruh·kayt

12. Salivary

Pronunciation link: <https://www.merriam-webster.com/dictionary/salivary>

IPA: /'sæl.ə.ver.i/

Phonetic Spelling: sal·uh·vair·ee

13. Pretreatment

Pronunciation link: <https://www.merriam-webster.com/dictionary/pretreatment>

IPA: /,pri:'tri:t.mənt/

Phonetic Spelling: pree·treet·muhnt

14. Cortisol

Pronunciation link: <https://www.merriam-webster.com/dictionary/cortisol>

IPA: /'kɔ:r.tə'sɔ:l/

Phonetic Spelling: kor·tuh·sawl

15. Syringe

Pronunciation link: <https://www.merriam-webster.com/dictionary/syringe>

IPA: /sə'rɪndʒ/

Phonetic Spelling: suh·rinj

16. SDS-PAGE

Pronunciation link: <https://www.howtopronounce.com/sds-page>

IPA: No confirmed IPA found

Phonetic Spelling: ess·dee·ess page

17. Lateral

Pronunciation link: <https://www.merriam-webster.com/dictionary/lateral>

IPA: /'læt.ə.əl/

Phonetic Spelling: lat·er·uhl

18. Assay

Pronunciation link: <https://www.merriam-webster.com/dictionary/assay>

IPA: /ə'seɪ/

Phonetic Spelling: uh·say

19. Migrate

Pronunciation link: <https://www.merriam-webster.com/dictionary/migrate>

IPA: /'maɪ.ɡreɪt/

Phonetic Spelling: my·grayt

20. ImageJ

Pronunciation link: <https://imagej.net/>

IPA: No confirmed IPA found

Phonetic Spelling: im·ij·jay

21. Rectangle

Pronunciation link: <https://www.merriam-webster.com/dictionary/rectangle>

IPA: /'rek.tæŋ.ɡəl/

Phonetic Spelling: rek·tang·guhl

22. Analyze
Pronunciation link: <https://www.merriam-webster.com/dictionary/analyze>
IPA: /'æn.əl.aɪz/
Phonetic Spelling: an·uh·lyze
23. Adjacent
Pronunciation link: <https://www.merriam-webster.com/dictionary/adjacent>
IPA: /ə'dʒeɪ.sənt/
Phonetic Spelling: uh·jay·suhnt
24. Normalized
Pronunciation link: <https://www.merriam-webster.com/dictionary/normalize>
IPA: /'nɔːr.məl.aɪzd/
Phonetic Spelling: nor·muh·lyzed
25. Turbidity
Pronunciation link: <https://www.merriam-webster.com/dictionary/turbidity>
IPA: /tə'brɪd.ə.ti/
Phonetic Spelling: ter·bid·uh·tee
26. Bradford
Pronunciation link: <https://www.merriam-webster.com/dictionary/Bradford>
IPA: /'bræd.fəd/
Phonetic Spelling: brad·ferd
27. Absorbance
Pronunciation link: <https://www.merriam-webster.com/dictionary/absorbance>
IPA: /əb'zɔːr.bəns/
Phonetic Spelling: ub·zor·buhns
28. Capillary
Pronunciation link: <https://www.merriam-webster.com/dictionary/capillary>
IPA: /'kæp.ə.lər.i/
Phonetic Spelling: kap·uh·lair·ee
29. Mucins
Pronunciation link: <https://www.merriam-webster.com/dictionary/mucin>
IPA: /'mjuː.sɪn/
Phonetic Spelling: myoo·sin
30. Upstream
Pronunciation link: <https://www.merriam-webster.com/dictionary/upstream>
IPA: /ˌʌp'striːm/
Phonetic Spelling: up·streem