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Title: The Nijmegen Hemostasis Assay: Simultaneous Fluorogenic Measurement of Thrombin and Plasmin Generation in a Single Well

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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**

NOTE: The SCs were shot by the videographer

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: 20

Number of Shots: 44

Introduction

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. **Bauke Haisma:** My research focuses on measuring secondary hemostasis and fibrinolysis together to better understand overall hemostatic balance.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Bauke Haisma:** Most current assays assess isolated components of coagulation or fibrinolysis. This protocol measures both processes simultaneously in one 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?~~

- 1.4. **Bauke Haisma:** The Nijmegen Hemostasis Assay is well suited for research into rare coagulation factor deficiencies, fibrinolytic disorders, and hemostatic drug effects.
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.5. **Bauke Haisma:** The main challenges are standardization of the technique and normalization of results to improve comparability across runs and laboratories.
 - 1.5.1. 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.~~

1.6. **Bauke Haisma**: Future work may focus on automating the assay and developing adaptations for compatibility with whole blood or platelet-rich plasma.

1.6.1. 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.

Ethics Title Card

This research has been approved by the Medical Ethics Committee of Arnhem-Nijmegen,
Radboud university medical center, Nijmegen, the Netherlands

Protocol

2. Quantifying Thrombin and Plasmin Generation Using a Fluorescence Plate Reader

Demonstrator:

NOTE: The demonstrators names were not provided

2.1. To begin, turn on the fluorescence plate reader and allow it to equilibrate to 37 degrees Celsius [1].

2.1.1. WIDE: Talent powering on the fluorescence plate reader and confirming the temperature setpoint on the display.

2.2. Configure the filter settings by setting the **number of wavelength pairs** to 2 [1]. Set the excitation wavelength to 355 nanometers for thrombin and 485 nanometers for plasmin [2]. Then set the emission wavelength to 460 nanometers for thrombin and 520 nanometers for plasmin [3].

NOTE: The SCREEN shots were filmed by the videographer

2.2.1. SCREEN: The number of wavelength pairs is being set to 2.

2.2.2. SCREEN: Talent entering excitation wavelengths for thrombin and plasmin.

2.2.3. SCREEN: Talent entering emission wavelengths for thrombin and plasmin.

2.3. Next, set the **plate type** to a 96 Well Greiner flat bottom black microplate [1]. Define the plate layout using a maximum of 20 wells per plate [2].

2.3.1. SCREEN: The plate type is set to a 96 Well Greiner flat bottom black microplate.

2.3.2. SCREEN: Talent highlighting selected wells in the plate layout editor.

2.4. Configure the **PMT and Optics** settings by setting the PMT gain to 200 volts and the number of flashes per read to 6 [1].

2.4.1. SCREEN: The PMT gain is set to 200 volts and the flashes per read to 6.

2.5. Then navigate to **Timing** to confirm the timing settings with a total run time of 1 hour 9 minutes and 30 seconds and interval of 30 seconds [1-TXT]. Navigate to **Shake** to confirm a shake of 3 seconds before the first read [2]. Navigate to **More settings** to ensure that the **Read order** is set to **Column** [3].

2.5.1. SCREEN: The run time is set to 1:09:30 and the interval is set to 00:00:30.

TXT: The number of reads is 140

- 2.5.2. SCREEN: Shaking before first read is selected and set to 3 seconds.
- 2.5.3. SCREEN: Talent navigating to More Settings and confirming read order 'Column'.
- 2.6. Configure the **Compound Transfer before Read** settings by setting the number of compound transfers to 1 and initial volume to 120 microliters [1]. Check to ensure that the **pipette height** is 130 microliters, **volume** is 20 microliters, **rate** is 1 microliters per second and **time point** is 14 seconds [2].
 - 2.6.1. SCREEN: The number of compound transfers is set to 1, with an initial volume of 120 uL.
 - 2.6.2. SCREEN: Talent checking the correct values of the other parameters pipette height (130 uL), volume (20 uL), rate (1 uL/sec) and time point (14 seconds).
- 2.7. Under **Compound Source**, confirm the 96-well compound plate format and specifically choose the **Beckman 96 2.3 milliliter** option [1]. Confirm the **Pipette tips layout** settings by selecting the correct columns [2].
 - 2.7.1. SCREEN: Talent selecting the 96 wells plates format and subsequently the specific Beckman 96 2.3 mL.
 - 2.7.2. SCREEN: Talent checking the correct pipette tips column layout settings.
- 2.8. Now navigate to **Compound & Tips Columns** and select the correct columns [1].
 - 2.8.1. SCREEN: Talent selecting the appropriate compound and tips columns.
- 2.9. Set the microplate shaker temperature to 37 degrees Celsius and verify the temperature before use [1]. Then set the shaking intensity to 1100 revolutions per minute [2-TXT].
 - 2.9.1. Shot of the microplate shaker temperature being set to 37°C.
 - 2.9.2. Shot of the shaking intensity being set to 1100 rpm. **TXT: Use the shaker for all subsequent mixing steps**
- 2.10. Next, thaw patient plasma and normal pooled plasma in a 37-degrees Celsius water bath for up to 10 minutes [1]. Mix the plasma samples thoroughly and verify complete thawing with no visible cryoprecipitate [2].
 - 2.10.1. Shot of labeled tubes of plasma being placed in a temperature-controlled water bath.
 - 2.10.2. Talent gently mixing plasma tubes and inspecting clarity.
- 2.11. Using a pipette, add 80 microliters of patient plasma or normal pooled plasma to the preselected wells, using a fresh pipette tip for each sample [1].
 - 2.11.1. Talent pipetting 80 µL plasma into designated microplate wells.
- 2.12. Then mix 48 microliters of cephalin with 48 microliters of tissue factor [1]. To this, add 240 microliters tris sodium chloride buffer [2]. Then pipette 96 microliters thrombin

substrate and 48 microliters plasmin substrate [3].

2.12.1. Talent pipetting 48 μ L cephalin and 48 μ L tissue factor into a microcentrifuge tube.

2.12.2. Talent adding 240 μ L of tris sodium chloride buffer to the tube.

2.12.3. Talent pipetting 96 μ L of thrombin substrate and 48 μ L of plasmin substrate into the tube and mixing again with the plate shaker.

2.13. Pipette out 20 microliters of the substrate mixture into each well [1]. Shake afterwards [2].

2.13.1. Talent pipetting 20 μ L of the substrate mixture to each well in the plate on the shaker.

2.13.2. Talent closing the lid of the shaker.

2.14. Now insert the microplate into the fluorescence plate reader [1-TXT].

2.14.1. Talent sliding the microplate into the reader tray. **TXT: Allow the plate to equilibrate at 37°C for several minutes**

2.15. Combine Tris sodium chloride buffer, Tris sodium chloride calcium chloride buffer, and tissue plasminogen activator to prepare the starting reagent [1]. Vortex thoroughly to mix [2]. Pipette the starting reagent mixture in all rows of the compound column [3].

2.15.1. Talent combining reagents in a tube.

AND

TEXT ON PLAIN BACKFROUND:

Reagent for Pump 1:

Tris/NaCl buffer: 672 μ L

Tris/NaCl/CaCl₂ buffer: 192 μ L

tPA: 96 μ L

Video Editor: Please play both shots side by side in a split screen

2.15.2. Talent vortexing the reagent tube.

2.15.3. Talent pipetting the starting reagent mixture in all rows of the compound column

2.16. Insert the plate containing starting reagent mixture to the source drawer [1] and start the fluorescence measurement [2]. Record fluorescence signals every 30 seconds for 70 minutes [3-TXT].

2.16.1. Talent placing the plate in the reader.

2.16.2. SCREEN: Measurement being initiated after manual addition.

2.16.3. SCREEN: Real-time kinetic fluorescence data appearing during acquisition. **TXT: Maintain plate at 37 °C throughout the measurement**

2.17. For data analysis, export the kinetic fluorescence data as a table containing timepoints

and fluorescence values per well [1-TXT]. ~~Convert the raw fluorescence data into enzyme generation curves by calculating the first derivative using spreadsheet or analysis software [2].~~ Plot the enzyme generation curves for each well to visualize thrombin or plasmin generation over time [3].

2.17.1. SCREEN: Talent exporting data from the plate reader software. **TXT: Calculate the 1st derivative**

~~2.17.2. SCREEN: The first derivative of the raw data is being calculated.~~

2.17.3. SCREEN: Final enzyme generation curves are being plotted.

Results

3. Results

- 3.1. Thrombin and plasmin generation curves from three healthy controls and normal pooled plasma showed reproducible peak height and timing [1], serving as reference profiles for normal hemostatic function [2].
 - 3.1.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the overlapping curves for "Healthy control 1", "Healthy control 2", "Healthy control 3"*
 - 3.1.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the overlapping curves for "Healthy control 1", "Healthy control 2", "Healthy control 3", and "Normal pooled plasma"*
- 3.2. In a patient with factor V (five) activity of 3%, thrombin generation was completely absent [1]. However, plasmin generation increased marginally [2].
 - 3.2.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the flat yellow curve labeled "FV activity 3%" in the thrombin graph.*
 - 3.2.2. LAB MEDIA: Figure 3D. *Video editor: Highlight the yellow curve labeled "FV activity 3%" in the plasmin graph.*
- 3.3. The patient with factor V activity of 44% showed reduced thrombin generation with a prolonged lag time and decreased peak height [1], while plasmin generation remained within the normal range [2].
 - 3.3.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the orange curve labeled "FV activity 44%"*
 - 3.3.2. LAB MEDIA: Figure 3D. *Video editor: Highlight the orange curve labeled "FV activity 44%" overlapping the "Normal pooled plasma" curve in the plasmin graph.*
- 3.4. In alpha-2-antiplasmin deficiency, plasmin generation increased with decreasing A2AP (Alpha-Two-Antiplasmin) activity [1], with the 23% activity sample showing an early onset of plasmin generation, a shorter fibrin lysis time, and a higher plasmin peak [2]. These alterations remained visible in the 73% activity sample, although not as pronounced as in the other sample [3].
 - 3.4.1. LAB MEDIA: Figure 3F. *Video editor: Sequentially highlight the three curves labeled "Normal pooled plasma," "A2AP activity 73%," and "A2AP activity 23%"*
 - 3.4.2. LAB MEDIA: Figure 3 E and F. *Video editor: Zoom in on the dark purple curve labeled "A2AP activity 23%" in both graphs*

3.4.3. LAB MEDIA: Figure 3E and F. *Video editor: Highlight the medium purple curve labeled "A2AP activity 73%" in both graphs*

Pronunciation Guide:

❏ Hemostasis

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

IPA: /ˌhi:mə'steɪsəs/

Phonetic Spelling: hee-muh-stay-suhs

❏ Fluorogenic

Pronunciation link: No confirmed link found

IPA: /ˌflʊərə'dʒenɪk/

Phonetic Spelling: floor-uh-jen-ik

❏ Thrombin

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

IPA: /ˈθrɒmbɪn/

Phonetic Spelling: throm-bin

❏ Plasmin

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

IPA: /ˈplæzmɪn/

Phonetic Spelling: plaz-min

❏ Nijmegen

Pronunciation link: No confirmed link found

IPA: /ˈnaɪmeɪɡən/

Phonetic Spelling: nye-may-guhn

❏ Fibrinolysis

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

IPA: /faɪˌbrɪnəˈlaɪsəs/

Phonetic Spelling: fy-brin-uh-lye-suhs

❏ Cephalin

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

IPA: /ˈkefəlɪn/, /ˈsefəlɪn/

Phonetic Spelling: kef-uh-lin; sef-uh-lin

❏ Cryoprecipitate

Pronunciation link: <https://www.merriam-webster.com/medical/cryoprecipitate>

IPA: /ˌkraɪəʊprɪˈsɪpətet/

Phonetic Spelling: kry-oh-prih-sip-uh-tayt

❏ Hemophilia

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

IPA: /ˌhi:məˈfɪliə/

Phonetic Spelling: hee-muh-fil-ee-uh

❏ Coagulation

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

IPA: /koʊˌæɡjəˈleɪʃən/

Phonetic Spelling: koh-ag-yuh-lay-shuhn

❏ Antiplasmin

Pronunciation link: No confirmed link found

IPA: / ,ænti'plæzmɪn/ , / ,æntaɪ'plæzmɪn/

Phonetic Spelling: an·tee·plaz·min; an·tye·plaz·min

❏ Plasminogen

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

IPA: /plæz'mɪnədʒən/

Phonetic Spelling: plaz·min·uh·jun

❏ Equilibrate

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

IPA: / ,i:kwə'laɪbreɪt/

Phonetic Spelling: ee·kwuh·lye·brayt

❏ Normalization

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

IPA: / ,nɔrmələ'zeɪʃən/

Phonetic Spelling: nor·muh·luh·zay·shuhn

❏ Comparability

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

IPA: / ,kəmpərə'bɪləti/

Phonetic Spelling: kom·pruh·bil·uh·tee