Measuring the Enzymatic Activity of Coagulation Factor VIIa Using a Fluorescent Substrate (Amidolytic Activity)

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Background: Coagulation Factor VIIa (FVIIa) is a trypsin-like serine protease whose enzymatic activity is dramatically increased when it binds to its protein cofactor, tissue factor (TF). *In vivo*, the 1:1 complex of FVIIa and TF initiates the blood clotting cascade by activating two serine protease zymogens (factors IX and X) via limited proteolysis. *In vitro*, it is very convenient to measure the enzymatic activity of FVIIa using chromogenic or fluorogenic substrates. In this protocol, the substrate is SN-17a, a commercially available substrate consisting of a tripeptide linked via an amide bond to a derivative of the fluorophore, 6-amino-1-naphthalene-sulfonamide (ANSN). (SN-17a is D-Phe-Pro-Arg-ANSNH-C_6H_{11}·2HCl; F.W. = 777.81) Cleavage of this amide bond (hence the name, amidolytic activity) liberates the fluorophore, whose fluorescence can readily be measured. Fluorescent substrates are more sensitive than chromogenic substrates, allowing the detection of lower enzyme concentrations than with chromogenic substrates. On the other hand, they require special instrumentation. (See separate protocol for measuring FVIIa amidolytic activity using chromogenic substrates.)

TF enhances the amidolytic activity of FVIIa from 10 to 100 fold, depending on the substrate. The K_d for binding of FVIIa to truncated, soluble tissue factor (sTF) or to full-length TF that has been incorporated into neutral phospholipid vesicles is ~2 to 5 nM. The K_d for binding of FVIIa to full-length TF that has been incorporated into phospholipid vesicles containing phosphatidylserine is less than 50 pM. In this assay, we want the enzyme concentration (that is, the concentration of assembled FVIIa:TF complexes) to be about 0.5 nM. So, we typically use 0.5 nM FVIIa and an excess of TF. This can be 20-50 nM sTF (or TF incorporated into pure phosphatidylcholine vesicles), or 1-2 nM TF that has been incorporated into vesicles composed of a mixture of phosphatidylserine and phosphatidylcholine (TF/PCPS). In this protocol, we assume you are using TF/PCPS.

Typical final reaction conditions (100 µl final volume per reaction):

FVIIa + TF/PCPS		
25	mM	HEPES pH 7.4
100	mM	NaCl
5	mM	$CaCl_2$
0.1%		Bovine Serum Albumin
0.5	nM	FVIIa
1	nM	TF/PCPS
100	µM	SN-17a (substrate)

Materials & Solutions

- **2× HBSAC** *(store at 4°C)*
  50 mM HEPES pH 7.4
  200 mM NaCl
  10 mM $CaCl_2$
  0.2% w/v Bovine Serum Albumin
  0.1% w/v NaN_3
- **SN-17a** is available from Haematologic Technologies. Note that this substrate is NOT specific for FVIIa, so it may not be suitable for measuring FVIIa activity in the presence of other proteases (for example, trying to assay FVIIa in plasma).
- **Factor VIIa** can be purchased from various suppliers, including American Diagnostica, Haematologic Technologies, and Enzyme Research Labs.
- **TF** is available from Calbiochem. We are not aware of any commercial source for **sTF**.
- We use round-bottom 96-well plates for the assay (black polystyrene plates; Corning/Costar #3792). We read the plates using a SpecraMAX Gemini XS fluorescent microplate reader from Molecular Devices.

Method

Make the master mix below at room temperature, just before use. (Scale this recipe up for the number of wells you will be using.) Add water and 2× HBSAC first, mix well, and then add the proteins last:

1.25× Master mix for FVIIa + TF/PCPS		
37.5 – (*x*+*y*)	µl	water
62.5	µl	2×HBSAC
x	µl	FVIIa (to give 0.625 nM final)
y	µl	sTF (to give 1.25 nM final)
100	µl	TOTAL

- Put 80 µl of the 1.25× master mix per well of a 96 well plate.
- Start reaction by pipetting 20 µl of 0.5 mM SN-17a substrate per well *(avoid bubbles!)*.
- Have the plate reader shake the plate one time for 5 sec, then monitor fluorescence using the following parameters (for the Molecular Devices SpectraMAX Gemini XS reader set to read *fluorescence (RFUs)* on the *kinetic* setting):
  - Wavelengths: 1
  - Excitation: 352 nm
  - Emission: 470 nm
  - Cutoff filter: 455 nm
  - PMT sensitivity: medium
  - Read once per min (6 reads/well) for 40 min total
- Record the initial rate of substrate hydrolysis. We usually perform these assays in duplicate at 37°C.

Notes

- Do not store either FVIIa or TF in the diluted state, as proteins are generally stable only when stored at high concentrations (~1 mg/ml or so). Make only the amount of master mix you can use in a single day. If you need to make intermediate dilutions of FVIIa or TF, make the dilutions in 1X HBSAC. It is important to keep the proteins in physiologic buffered saline with carrier BSA at all times. Never dilute them in water or buffer without carrier protein. Discard any excess diluted TF or FVIIa.
- FVIIa is a rather sticky protein, so we generally use SlickSeal pre-siliconized microcentrifuge tubes (MG Scientific) for storing or handling solutions containing FVIIa.
- On the day of use, dilute SN-17a substrate in water to give a 500 µM working solution.
- You can modify the protocol to vary one or more of the components. Make the appropriate strength master mix missing one or more of the components, then add master mix and the other (varied) component to each well to give 80 µl total/well. For example, you could add the following per well: 60 µl of an appropriate master mix plus 20 µl of the missing component. Start the reactions with 20 µl/well of 500 µM SN-17a substrate as above. You will get more reproducible results if you avoid pipetting multiple small volumes into each well.

References

Butenas S, DiLorenzo ME, and Mann KG. Ultrasensitive fluorogenic substrates for serine proteases. *Thromb. Haemost.* **78:**1193-201, 1997. [PubMed link]

Neuenschwander PF, Branam DE, and Morrissey JH. Importance of substrate composition, pH and other variables on tissue factor enhancement of factor VIIa activity. *Thromb. Haemost.* **70:**970-977, 1993. [PubMed link]