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

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http://tf7.org/mlabprotocols.htm

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 Chromozym t-PA, a commercially available substrate consisting of a tripeptide linked via an amide bond to the dye, *p*-nitroaniline ($MeSO_2$-D-F-G-R-*p*NA). Cleavage of this amide bond (hence the name, amidolytic activity) liberates the dye, which then absorbs light at 405 nm.

TF enhances the amidolytic activity of FVIIa from 10 to 100 fold, depending on the substrate. Reasonable concentrations of FVIIa to use in this amidolytic assay are 50 nM FVIIa in the absence of TF, or 5 nM FVIIa in the presence of saturating levels of TF. 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. So, we typically use 50-100 nM sTF (or TF incorporated into pure phosphatidylcholine vesicles), or 10 nM TF that has been incorporated into vesicles composed of a mixture of phosphatidylserine and phosphatidylcholine. In this protocol, we assume you are using sTF.

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

FVIIa alone		
25	mM	HEPES pH 7.4
100	mM	NaCl
5	mM	$CaCl_2$
0.1%		Bovine Serum Albumin
50	nM	FVIIa
1	mM	Chromozym t-PA (substrate)

FVIIa + sTF		
25	mM	HEPES pH 7.4
100	mM	NaCl
5	mM	$CaCl_2$
0.1%		Bovine Serum Albumin
5	nM	FVIIa
100	nM	sTF
1	mM	Chromozym t-PA (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

- **Chromozym t-PA** is available from Roche Diagnostics. Other chromogenic substrates that are suitable for measuring FVIIa amidolytic activity include **CBS34.47** from Diagnostica Stago; **S-2266**, **S-2288**, **S-2238** or **S-2366** from diaPharama/Chromogenix; or **Spectrozyme fVIIa** or **Spectrozyme TH** from American Diagnostica. Note that NONE of these substrates are specific for FVIIa, so they may not be suitable for measuring FVIIa activity in the presence of other proteases (for example, trying to assay FVIIa in plasma).
- Fluorescent substrates for measuring FVIIa activity are available from Haematologic Technologies and are more sensitive than chromogenic substrates. (See separate protocol for measuring FVIIa enzymatic activity using fluorescent substrates.)
- **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**.
- Use polystyrene, flat-bottom, 96-well plates for the assay. (We use Ultra Low Attachment plates; Corning/ Costar #3474.) We read A_{405} using a VERSAmax microplate reader from Molecular Devices.

Method

Make the master mixes below at room temperature, just before use. (Scale these recipes 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 alone	**1.25× Master mix for FVIIa + sTF**
37.5 – *x* µl water	37.5 – (*x*+*y*) µl water
62.5 µl 2×HBSAC	62.5 µl 2×HBSAC
x µl FVIIa (to give 62.5 nM final)	*x* µl FVIIa (to give 6.25 nM final)
	y µl sTF (to give 125 nM final)
100 µl TOTAL	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 5 mM Chromozym t-PA substrate per well *(avoid bubbles!)*.
- Have the plate reader shake the plate one time for 5 sec, then monitor A_{405} in a microplate reader on *kinetic* setting, reading the plate every 30 sec for 20 min. (Try using an OD limit of 0.2.) Record the initial rate of substrate hydrolysis. We usually perform these assays in duplicate at room temperature.

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.

- Typically, Chromozym t-PA is dissolved in water to give a 5 mM solution. Store the Chromozym t-PA stock at –20 or –80°C. It's expensive, so don't waste it!

- 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 5 mM Chromozym t-PA as above. You will get more reproducible results if you avoid pipetting multiple small volumes into each well.

- You can use this protocol to measure the level of TF or FVIIa in an unknown solution. For example, to use this protocol to measure TF levels, make a standard curve with varying concentrations of TF and a fixed amount of factor FVIIa (typically, 5 nM). You can then compare the activity of the unknown to this standard curve. You can also use this assay to measure the concentration of full-length TF in detergent (such as octyl glucoside or Triton X-100). Just make sure you prepare the standard curve in the same detergent.

Reference

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]