S-2288 tPA Protocol
Microplate Method

Example protocol only.
S-2288 is for laboratory use only, not for diagnostic purposes.

The Chromogenix S-2288 Assay reliably quantifies the amidolytic activity of the tPA bound to product. tPA hydrolizes tripeptide chromogenic substrates resulting in p-nitroaniline that absorbs at 405nm. The production of pNA is tracked through time and its rate of production in the first five minutes is averaged using its absorbance at 405nm. This activity is proportionate and allows a mathematically predictable activity of the tPA present in the 3-30U/L range. Assuming no free tPA is in the supernatant (after multiple washes), allows the product/tPA amidolytic activity to be quantified directly from the assay. Amidolytic activity and fibrinolytic activity are not neccesarily parallel, but conclusions may be drawn using the amidolytic activity in combination with lytic rates, and total protein concentrations derived from further testing.

Materials and Equipment

- Lab notebook and Timer
- Chromogenix S-2288 Substrate
- 96 well plates
- Spectrophotometer with plate heater set to 37°C, λ=405nm
- Commercial Free tPA in solution, used as standard
- Eppendorf tubes
- Pipetters and tips
- Product in solution of water or PBS (known concentration of product and supernatant ID)
- DI Water or PBS for dilutions and blanks
- Safety wear, eyewear, gloves, labcoat

1. Reconstitute S-2288 substrate with 8.65ml filtered DI water.
2. Prepare a 0.01% Tween solution to use as a diluent for “unknown” product.
3. Reconstitute 3.4mg of Activase®(alteplase) directly from package in 3.0ml DI water(Activase® contains Tween as an ingredient). This will be tPA “known” stock. Pipette up and down or swirl gently to mix (*no vortexing*). Alternately tPA ”known” stock(.005028mg/ml) can be used from Bradford Assay if less than two days since reconstitution.
4. Dilute tPA “known” stock to 30U/L and 3U/L solutions as in example:

$$0.005028mg/ml(580{,}000U/\text{mg}) = 2916.3\text{U/ml}$$
$$2916.3\text{U/ml stock } (1.05\mu l/1001.05\mu l) = 3.059U/ml \text{ or } 3059U/L$$
$$3059U/L(9.8\mu\text{l}/1000.8\mu\text{l}) = 30\text{U/L}$$
$$30U/L(100\mu\text{l}/1000\mu\text{l}) = 3\text{U/L}$$

5. Note precise mass and resultant activity calculations in lab notebook with date and activity on tube label.
6. Maintain reconstituted lytic agents on ice, but not frozen.
7. Prepare Tris Buffer with the following proportions:

Tris	242mg
NaCl	124mg
DI water	16ml

   Adjust the pH to 8.4 by adding 1M HCl.(≈2ml). Btv to 20ml w/ DI water. Buffer is stable for 6months stored at 4°C.
8. Pipette 620µl product from package into a labelled Eppendorf Tube.
9. Pipette 610µl/sample (3X accounted for) + 200µl/each blank of Chromogenix S-2288 Substrate into labelled Eppendorf Tubes and cover with foil to protect from light during transport.
10. Prepare a 96 well plate map and tape in lab notebook as a legend.
    Note: Plate and map of Total Protein and Activity Assay can be combined, but Bradford Assay(Total protein) must be run first to assure room temperature reagents.
11. Pipette 20µl Tris buffer, pH8.4 into each well to be used as noted by map and cover to prepare for transport.
12. Add tPA “known” (100% and 10%) in triplicate to map.
13. Add Product “unknown” in triplicate to map.

96 Well Plate Map:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

14. Set λ to 405nm and Temperature to 37°C on Spectrophotometer.
15. Insert plate with 200µl Buffer in the appropriate wells.
16. Incubate at 37°C for 4 min.
17. After 4 minutes add 200µl sample (product, tPA, One Blank) pipetting up and down, to the appropriate wells from the map legend.
18. Incubate at 37°C for 4 min.
19. Quickly add 200µl of 37°C substrate to each well to be tested, pipetting up and down (very carefully) to mix.
20. Immediately insert drawer back into spectrophotometer and measure to get an absorbance at T_0.
21. Re-measure absorbance of each well every minute for five minutes, recording on table

Sample ID	Location	Absorbance (AU) at 405nm, 37°C					
		Initial	1 minute	2 minute	3 minute	4 minute	5 minute
Blank							
	a)						
	b)						
	c)						
	a)						
	b)						
	c)						
	a)						
	b)						
	c)						

22. Record the difference between 5minute and Initial absorbance and divide by five.
23. Obtain an average for each sample and calculate relative standard deviation.
24. Plot a line between the 10% and 100% tPA standards and extrapolate the bound tPA activity for the sample from this line.
25. Assure relative standard deviation <5%, that blank has no absorbance, and that sample falls within the test limits prior to reporting results.
26. Calculate the undiluted protein concentration.