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OLIGOBIND® Thrombin activity assay

Product no. ADG844

Storage: 2–8°C For Research Use Only!

PRODUCT INSERT ENGLISH

INTENDED USE

The OLIGOBIND® Thrombin activity assay is an enzyme-capture-assay for the quantitative measurement of active thrombin in stabilized plasma samples.

EXPLANATION OF THE TEST

The conversion of prothrombin to thrombin is a key event in thrombus (clot) formation. Thrombin is a serine protease that acts on a wide variety of substrates in the coagulation pathway. In vivo generated thrombin can be indirectly assessed by the measurement of prothrombin fragment F1.2, an activation peptide generated during the conversion of prothrombin to thrombin, or thrombin-antithrombin-complexes (TAT), formed during the inactivation of thrombin by its major plasma inhibitor antithrombin. However, due to differential accumulation in the circulation, these parameters do not reflect the current state level of functional active thrombin in vivo. In combination with the Thrombin blood collection tubes (Product No. ADG844T) that ensure the stabilization of thrombin-activity ex vivo, the OLIGOBIND® Thrombin activity assay allows the direct quantification of functional active thrombin in plasma from peripheral blood.

PRINCIPLE OF THE METHOD

Stabilized plasma samples are added to microwells coated with a DNA-aptamer against thrombin. During an incubation period, thrombin present in the sample will bind to the aptamer coated to the wells. Following a washing step, a fluorogenic peptide substrate for thrombin is added to the microwells. Measuring the change of fluorescence ($360_{[ex]}/460_{[em]}$ nm) and extrapolating the value with those of a standard curve determines the level of thrombin in the plasma sample.

REAGENTS

MTP	Aptamer Coated Microtiter plate, MTP-96 (12x8) well
WASH	Wash buffer, 50 ml, 1 vial (20x concentrate)
DILB	Sample dilution buffer, 2 ml, 1 vial (ready-to-use)
STD-40	Thrombin Standard plasma 1, 40.0 mU/ml, 0.5 ml, 2 vials (lyophilized)
STD-20	Thrombin Standard plasma 2, 20.0 mU/ml, 0.5 ml, 2 vials (lyophilized)
STD-4	Thrombin Standard plasma 3, 4.0 mU/ml, 0.5 ml, 2 vials (lyophilized)
STD-0.8	Thrombin Standard plasma 4, 0.8 mU/ml, 0.5 ml, 2 vials (lyophilized)
STD-0.16	Thrombin Standard plasma 5, 0.16 mU/ml, 0.5 ml, 2 vials (lyophilized)
STD-0	Thrombin Standard plasma 6, 0 mU/ml, 0.5 ml, 2 vials (lyophilized)
TSUB	Thrombin Substrate, fluorogenic substrate, 120 µl, 1 vial (100x concentrate), store in the dark!
SBUF	Substrate buffer, 12 ml, 1 vial (ready-to-use)

PRECAUTIONS

Source material for some of the reagents in this kit is of human origin. This material has been found to be non-reactive for Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus Type 1 and Type 2 (HIV-1, HIV-2) using FDA approved methods. As no known test method provides complete assurance that products derived from human blood will not transmit HBsAg, HCV, HIV-1, HIV-2 or other blood-borne pathogens, reagents should be handled as recommended for any potentially infectious human specimen. Discard all waste associated with test specimens and human source reagents in a biohazard waste container.

For *in vitro* use only. Not for internal use in humans or animals. Do not use the kit components beyond the stated expiration date. Do not mix reagents from different kits. Avoid microbial contamination of the reagents. Do not smoke, eat or drink in areas in which specimens or kit reagents are handled. Do not pipette reagents by mouth. Wear laboratory coat and disposable gloves throughout the test procedure and wash hands thoroughly afterwards. Avoid splashing or aerosol formation.

REAGENT PREPARATION AND STORAGE

Unopened and lyophilized reagents are stable until the expiration date printed on the box when stored as instructed.

MTP **Aptamer coated microwells:** Once removed from the foil pouch, the microwell strips must be used within 30 minutes. Unused strips may be stored at 2°-8°C for 4 weeks when sealed in the original pouch with the desiccant present, protected from any moisture.

WASH **Wash buffer:** Transfer the content to a 1 liter bottle and fill up the concentrate to 1 liter with filtered deionized/distilled water. Diluted Wash Buffer may be used for up to 4 weeks when stored at 2°-8°C.

DILB **Sample dilution buffer:** Supplied ready to use. Opened Sample dilution buffer is stable for 3 month when stored at 2°-8°C.

SBUF **Substrate buffer:** Supplied ready to use. Opened Substrate buffer is stable for 3 month when stored at 2°-8°C.

STD **Standards:** Reconstitute each Thrombin standard plasma with 500 µl purified, deionised or distilled water, swirl the contents gently and allow the vials to stand at room temperature for at least 15 minutes to ensure complete dissolution. The lyophilised plasma is stable until the date indicated on the vial label when stored at 2° - 8°C.

TSUB **Thrombin Substrate:** Supplied as a concentrate, dilute the Thrombin Substrate 1:100 with Substrate Buffer just prior to use. For running all 96 microwells at one time, dilute 100 µL of Thrombin Substrate to 10 mL in Substrate Buffer. If not all 96 microwells are used, dilute 10 µL of Thrombin Substrate to 1 mL in Substrate Buffer for each 8-micro-well strip that will be used. Working strength Thrombin Substrate is stable for 2 hours at 2°-8°C. Discard any unused working strength Thrombin Substrate. Opened Thrombin substrate should be stored in the dark at -20 °C.

SPECIMEN COLLECTION AND PREPARATION

Plasma prepared from peripheral blood collected in Thrombin blood collection tubes (Product No. ADG844T) should be used for this assay. For general sample handling, see "Collection, Transport and Processing of Blood Specimens for Testing Plasma-based Coagulation Assays; Approved Guidelines-Fourth Edition", NCCLS Document H21-A4, Vol. 23, No. 35, December 2003.

Plasma collection should be performed as follows:

1. Collect blood into Thrombin blood collection tubes.
2. Drawn blood should be stored at *room temperature* and centrifuged (see step 3) within 4 hours.
3. Centrifuge the blood sample at 2,500 x g for 15 minutes.
4. Plasma should be stored at 2°-8°C and assayed within 4 hours. Alternatively, plasma may be stored at -40°C for up to 6 months.
5. Frozen plasma should be thawed rapidly at 37°C.

PROCEDURE

Materials Provided – See Reagents

Material Required But Not Provided

0.22 µm filtered deionized H_2O
50-300 µL eight channel multi-pipette
0-200 µL, 200-1000 µL single pipettes
microwell plate reader for reading fluorescence at $360_{[ex]}/460_{[em]}$ nm
microwell plate washer

Preparing the Thrombin Standards

1. Reconstitute the Thrombin standard plasmas as instructed under REAGENT PREPARATION AND STORAGE.

Sample Dilutions

2. The reconstituted Thrombin standards are ready-to-use. Do not dilute. Also the plasma samples should initially be tested undiluted.

Running standard and samples in duplicate is recommended.

Assay Procedure

3. Open the foil pouch and remove the microwell strips/frame assembly. Remove the strips that will not be used, return them to the foil pouch and tightly reseal the pouch with the desiccant inside. Store the foil pouch at 2 - 8°C.
4. Pipette 100 µL of the standards or samples into separate microwells, cover with the acetate sheet and incubate for 1 hour at room temperature (20-25°C) in the dark.
5. Prepare the Thrombin substrate working solution as instructed under REAGENT PREPARATION AND STORAGE. Empty the contents of the microwells with an eight channel multi-pipette using fresh tips for each strip. Subsequently, manually add 250 µl of Wash Buffer to each microwell and wash additional 3 times with Wash Buffer (300 µl / microwell). Remove any remaining droplets by tapping the plate 4-5 times, face down against absorbing material.
6. Place the microwell plate in a fluorescence plate reader set at $360_{[ex]}/460_{[em]}$ nm and the temperature set at 20-25 °C.

Measurement (kinetic method)

7. Add 100 µL working strength Thrombin Substrate to each microwell. The reaction begins immediately upon addition of the substrate. Measure fluorescence at room temperature at $360_{[ex]}/460_{[em]}$ nm within 1-2 minutes (T=0).
8. Measure the increase in fluorescence over 30 minutes, collecting data at 5 minutes intervals. Take the linear part of the curve and calculate the rate of change in fluorescence (dFU/min).

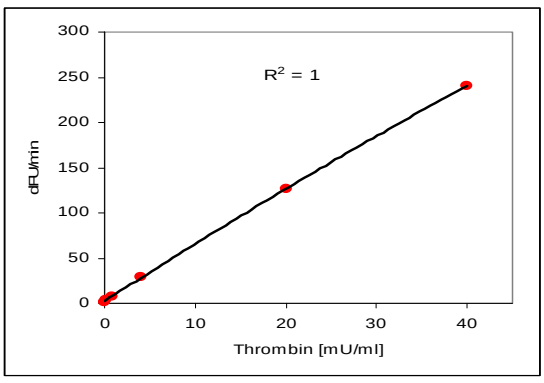
Alternative measurement (endpoint method)

7. Add 100 µL working strength Thrombin Substrate to each microwell. The reaction begins immediately upon addition of the substrate, measure fluorescence at room temperature at $360_{[ex]}/460_{[em]}$ nm within 1-2 minutes (T=0).
8. Read the fluorescence again after 30 min (T=30). Calculate the net change in fluorescence, dFU (T30 - T0).

RESULTS

A standard curve is constructed by plotting the mean change of fluorescence (dFU) for each standard versus the corresponding concentration of Thrombin in mU/mL. A standard curve should be generated each time the assay is performed. A representative standard curve using a Fluostar Optima Fluorometer (BMG Labtech) is shown below and is for demonstration purposes only.

Representative Standard Curve



CALCULATIONS

Use an appropriate curve fit software or calculate the amount of Thrombin in the plasma sample by interpolating directly from the standard curve.

Samples which yield values above the highest standard must be pre-diluted and retested. Sample dilutions (e.g 1:2) must be prepared using the provided Sample dilution buffer.

When the plasma sample was diluted, multiply the results by the dilution factor (e.g. 2) in order to obtain the concentration of Thrombin in the neat plasma sample. The calculation is:

$$[\text{Thrombin}]_{\text{Plasma Sample}} = [\text{Thrombin}]_{\text{Diluted Test Sample}} \times 2$$

LIMITATIONS OF THE PROCEDURE

Platelet contamination in plasma samples may interfere with the assay results. Plasma samples must be free of platelets in order to have a valid result. Exercise great care in minimizing disruption of the platelet pellet while recovering the platelet poor plasma. Samples should not be frozen and thawed more than once.

EXPECTED VALUES

In a study using the Oligobind® Thrombin activity assay in combination with Thrombin blood collection tubes the thrombin concentration in the plasma from normal adult donors (n=20) was determined to be < 0.35 mU/ml (below the lower limit of quantification [LLOQ] of the assay).
In samples taken during the course of hip replacement surgeries, plasma thrombin concentrations were found to be increased.[2]

PERFORMANCE CHARACTERISTICS

Sensitivity

For plasma samples, the LOD was found to be 0.10 mU/ml thrombin while the LLOQ was determined as 0.35 mU/ml thrombin.

Precision

The mean intra- and inter-assay coefficients of variations (CV) for this assay as determined at different plasma input concentrations of thrombin have been estimated to be 6.9 ± 0.6 % and 6.5 ± 3.1 % respectively.

Specificity

The assay is specific for thrombin in human plasma samples. Assay performance on thrombin from other species has not been tested.

BIBLIOGRAPHY

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3. Measurement of free thrombin in human plasma using an oligonucleotide-based enzyme capture assay (OECA). Becher T, Müller J, Braunstein J, Mayer G, Pötzsch B. Haemostasiologie, 2010, 30 1: P17-02.