AGP TEST

For *in Vitro* diagnostic use only

Test for the evaluation of platelet aggregation induced by ADP, Adrenaline, Collagen and Ristocetin

I. INTENDED USE

ADP, Adrenaline, Collagen and Ristocetin are for use in routine platelet aggregation studies for the evaluation of platelet dysfunction or platelet activation.

II. PRINCIPLE

The study of the aggregation on PRP is based on the evaluation of changes in transmittance in a plasma sample during the aggregation.
When ADP, Adrenaline and Collagen are added to platelet rich plasma, it stimulates platelets to change shape and to aggregate. The aggregation induced by exogenous ADP is primary and reversible aggregation. The aggregation induced by Adrenaline is primary aggregation. For the collagen there is a lag phase during which the collagen fibrils polymerize for platelet activation. The normal platelets release endogenous ADP from its granules. The release of endogenous ADP occurs in a second wave of aggregation.
The ristocetin, a concentration of 1.0-1.5 mg/ml, normal platelet aggregates in rich plasma or citrated whole blood through a mechanism in which the release of endogenous ADP plays only a small role. Ristocetin-induced platelet aggregation at concentrations of 1.2 mg/ml is absent or significantly lower in patients with von Willebrand's syndrome. At concentrations of 1.5 mg/ml there is a lower degree of abnormality. The majority of patients with von Willebrand's syndrome shows a negative response, as well as patients with Bernard-Soulier syndrome.
Aggregation study in whole blood is based on to the evaluation of the electric resistance changes. Two electrodes while immersed in the sample are rapidly covered with platelets which, at the very first contact, appear in shape of monolayer. When the aggregating agent is added, additional platelets tend to attach the monolayer previously formed, determining an impedance increase between the two electrodes.

III. REAGENTS AND MATERIALS

Each kit contain:

1. ADP 0,1 mM lyo (2 vials x 0,5 mL)
2. Lyophilized 5 mM Adrenaline (5 vials x 0,5 mL)
3. Collagen 1 mg/mL (1 vial x 0,5 mL): suspension with 1 mg/mL of Type 1 collagen fibrils sourced from equine tendon, type I
4. Ristocetin 25 mg (1 vial x 0,5 mL): Ristocetin A sulphate lyophilised form. Antibiotic isolated from Nocardia lurida, containing in excess of 90% Ristocetin A
5. Diluent A (1 vial x 50 mL): buffer for dilution with TRIS, pH 7,3
6. Diluent B (1 vial x 4,0 mL): solution to dilute collagen
7. Instruction for use.

MATERIAL REQUIRED BUT NOT SUPPLIED

- Blood collection tubes, centrifuge tubes, tubes and pipettes for drawing up the blood and the PRP, all in siliconized glass or plastic.
- Bidistilled water.
- Aggregometer.

IV. STORAGE and REAGENTS PREPARATION

Store the kit at +2 - +8°C,The kit is stable until expiration date printed on the package label.
ADP - Reconstitute one vial with 0,5 ml of Diluent A. The lyophilized reagent once reconstituted is stable for 8 hours at RT, 2 weeks at +2 - +8°C and 1 month at -20°C. To avoid repeated thawing and freezing it is advised to subdivide the solution into aliquots of 0,1 ml and freeze. With the work scheme proposed here, the reagent is sufficient to carryout **70 aggregation test/vial** (generally about 35 patients/vial).
Adrenaline – Reconstitute one vial with 0,5 mL of distilled water. After reconstitution and taking of the amount required for the test, the vials of **adrenaline** should be removed. Before use dilute two a 0,1 ml aliquots of **Adrenaline** (previously reconstitute) with 4,9 ml and 0,9 ml of **Diluent A** to obtain two working solutions at concentrations of 0,1 mM and 0,5 mM respectively. Working solution is stable for 60 minutes at RT. Each vial contains sufficient reagent to carry out **100 aggregation test.**
Collagen - Mix by inverting the collagen MB and dilute 0.1 ml with 0.4 ml of diluent MB. Concentration of work solution: 200 µg/ml. This solution is stable few hours in a bath of melting ice.
Ristocetin - reconstitute a vial of Ristocetin with 0,5 ml of Diluent A. Concentration of work solution: 50 mg/ml. This solution is stable one month at -20°C. To avoid repeated thawing and freezing it is advised to subdivide the solution into aliquots of 0,1 ml and freeze. With the work scheme proposed here, the reagent is sufficient to carryout **33 aggregation test/vial.**

V. SAMPLE COLLECTION

Collect the sample from an antecubital vein without stasis by slowly drawing up the blood with the syringe and slowly expelling it (after having removed the needle), into the collection tube; avoid haemolysis. Carry out the venepuncture with a plastic syringe and mix 9 volumes of blood with 1 volume of trisodium citrate 3.8% in a plastic or siliconized tube.
If the aggregation is carried out onto PRP, respect the following instruction:

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Centrifuge the blood at 200 g for 10 minutes, carefully draw off the supernatant (PRP) and carry out a platelet count on this. Re-centrifuge the remaining citrated blood at 2000 g for 30 minutes and decant the supernatant (PPP). Dilute PRP with PPP to obtain a plasma with about 300.000 platelet/mm^3. Maintain the PRP at room temperature and carry out the test within 4 hours.

VI. TEST PROCEDURE

The following working plan is for a volume of 0,5 mL of PRP (in agreement with the type of aggregometer) or 1 mL of a blood.
Transfer 0,5 ml of PRP and 0,5 ml of PPP into two cuvettes of the aggregometer, previously prepared for the test according to the manufacturer's instructions and set each channel to 90% and 10% trasmittance respectively with the cuvettes of PPP and PRP. The setting must be repeated for each plasma and for each cuvette.
The operating procedures proposed here for the various aggregant agents are based on the results reported in the literature for normal subjects. It is therefore advisable that each laboratory establish its own normal range and follow a work scheme for carrying out the test which takes into account the obtained values.
ATTENTION: with the new instruments it is possible carry out a fast and simple process of auto-calibration (see the operatives manual for each instrument). This procedure allows a better standardization of the used method.

VII. PRP PROCEDURE

1. Prepare PRP and PPP as described in section V.
2. Add 500 µl (250 µl) of PRP to an aggregation cuvette containing stirring bar and incubate at 37°C for 3 minutes.
3. Add 500 µl (250 µl) of PPP to an aggregation cuvette without stirrer.
4. Place PRP and PPP cuvettes in corresponding instrument sample wells and follow manufacturer's instruction for setting base lines.
5. Bring aggregating reagents to room temperature and swirl to mix.
6. For each aggregating reagent add the quantity indicated in the table to PRP cuvette to obtain different aggregation curves.
7. Record platelet aggregation response for a minimum of 5 minutes.

Parameter	Concentration	Aggregation type	volume to add at PRP	Notes
ADP	0.8 µM	biphasic	4.0 µL (2.0 µL) *	Routine test
	2 µM	irreversible monophasic	10.0 µL (5.0 µL) *	
Adrenaline	1.0 µM	biphasic	5.0 µL (2.5 µL) * og Adrenaline 0.1 mM	Routine test
	10 µM	irreversible monophasic	10.0 µL (5.0 µL) * of Adrenaline 0.5 mM	
Collagen	200 µg/mL		10.0 µL (5.0 µL) *	
Ristocetin	1.5 mg/mL		15.0 µL (7.5 µL) *	
If an aggregation is not obtained or is markedly reduced, (von Willebrand's disease and Bernard-Soulier's syndrome) repeat the test, in order to have a confirmation of the diagnosis, operating as follows: - to 0,4 ml of PRP add 0,1 ml of normal pooled plasma; - add 15 µl of Ristocetin to obtain a final concentration of 1,5 mg/ml; If an increase in aggregation is recorded, the diagnosis of von Willebrand's disease is confirmed.				

* The figures in parentheses are half volumes that a lot of aggregometers can now handle; using the proper rubber adhesive spacers.

VIII. WHOLE BLOOD PROCEDURE

1. Add 500 µl of saline solution and 500 µl of whole blood with anticoagulant in a 1 ml plastic cuvette containing stirring bar and incubate at 37°C for 5 minutes.
2. After connecting the electrode to the socket, put incubated at 37°C for 5 minutes.
3. Alfter incubation, place it in the vial containing the diluted blood. (Place the filaments to the back of aggregometer).
4. Place the cuvette into the reaction well and incubate 2 minutes, holding the door closed. Open the door and pipette the amount of collagen careful pipette on the bottom, rinse several times or 20 µL of reconstituted Ristocetin (Warning: avoid formation of air bubbles).
5. Record platelet aggregation.

Parameter	Finale concentration	volume to add at whole blood	Notes
Collagen	2 µg/mL	10 µL	It is recommended to work at two different concentrations of collagen: 2 µg/mL and 4 µg/mL
	4 µg/mL	20 µL	
	5 µg/mL	25 µL	
Ristocetin	1 mg/mL	20 µL	

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IX. INTERPRETING THE RESULTS

Normal values

As the normal absolutes values are not available yet, for whole blood aggregation, it is recommended for each laboratory to establish their own normal ranges in order to compare with aggregation curves taken from pathological subjects.

NOTE: The following Normal Ranges were obtained from various laboratories and pubblications. They should be used as a **guideline only.**

ADP	Concentrations < a 0,3 µM	reversible aggregation
	Conc. between 0,3 and 1,5 µM	biphasic aggregation
	Concentrations > a 1,5 µM	irreversible monophasic aggregation
	Concentration 5 µM	% aggregation 69 – 88 %
	Concentration 10 µM	% aggregation 71 – 88 %
Adrenaline	Conc. < 0,2 µM	reversible aggregation
	Conc. between 0,2 and 5,0 µM	biphasic aggregation with a second wave induced by endogen aggregating agents
	Conc > a 5,0 µM	irreversible monophasic aggregation **(% di aggregation 70-80%)**
Collagen on PRP	Concentration 2 µg/mL	% aggregation 70 - 94%
on whole blood	Concentration 2 µg/mL	aggregation (ohm) 15 – 27
	Concentration 5 µg/mL	aggregation (ohm) 15 – 31
Ristocetin on PRP	Concentration 1,5 mg/mL	% aggregation max 82 - 96%
on whole blood	Concentration 1,0 mg/mL	aggregation (ohm) >5 Ω. <70 sec Lag time

X. PERFORMANCES

This product will perform as described prior to its expiration date when procedural and storage directions are followed.

Linearity, accuracy, precision.

Platelet aggregation induced by common aggregating reagents (ADP, Arachidonic Acid, Collagen, Ristocetin and Adrenaline) is a nonlinear test system for some parameters: Lag Phase, Primary Slope, Secondary Slope, biphasic response and disaggregation. The non-linearity is caused by many factors such as the reaction chemistry and instrumentation. Platelet aggregation measures a response rate or activity that is not a quantitative measure of the reactants or their concentration.

In platelet aggregation, accuracy is a relative parameter and is dependent on the test system.

The limitations of platelet aggregation make it difficult to provide typical precision or reproducibility ranges.

XI. NOTE

- **Epinephrine is not recommended as a standard agonist for whole blood testing clinically**. As a fewer than 50% respond to this very weak agonist.
- To test at the same time optical test on PRP and the release of ATP with **bioluminescent** technique should work on a **lumi-aggregometer**. (Example 700-2). Refer to the Technical Manual and the instructions in User Manual of instrument.

XII. REFERENCES

Refer to the Technical Manual **M3115xxx BE-0 07/11**

CONTENT	**REF. 3115001 (900 tests)**
ADP 0,1 mM lyo	2 x 0,5 mL
Adrenaline 5mM lyphilized	5 x 0,5 mL
Collagen 1 mg/mL	1 x 0,5 mL
Ristocetin 25 mg	1 x 0,5 mL
Diluent A	1 x 50 mL
Diluent B	1 x 4,0 mL
Instruction for use	1 item

IVD	In Vitro Diagnostic Medical Device		Temperature limitation	LOT	Batch code (LXXX)		Manufacturer		Keep dry	NON STERILE	Non-sterile
	Consult Instructions for use		Use by (year/month)	REF	Catalogue number		Do not reuse		Fragile, handle with care		Keep away from heat

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