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CHRONO-LOG CORPORATION

CHRONO-PAR® AND CHRONO-LUME® REAGENTS FOR PLATELET FUNCTION TESTING & SECRETION STUDIES IN WHOLE BLOOD AND PLATELET RICH PLASMA

(For In-Vitro Diagnostic Use)

INTRODUCTION

CHRONO-PAR® and CHRONO-LUME® reagents are used to confirm normal platelet function and to diagnose platelet dysfunctions.

The following CHRONO-PAR and CHRONO-LUME reagents are suitable for use in both Whole Blood and Platelet Rich Plasma:

ADP (P/N 384) – In PRP, with low concentrations, (< 1μM), shape change is followed by primary aggregation and disaggregation. At higher concentrations of 1-5 μM a biphasic response is often visible. Second wave aggregation requires the synthesis of thromboxane A2 and is affected by cyclooxygenase inhibitors such as aspirin. Aggregation with ADP in Whole Blood requires higher concentrations of ADP (typically 20 μM).

Arachidonic Acid (P/N 390) – A direct test for prostanoid synthesis, as aggregation requires conversion to thromboxane A2 by cyclooxygenase, a process which is

inhibited by aspirin. Responses between no aggregation and the normal range frequently indicate drug ingestion some time during the previous days.

ATP Standard (P/N 387) – For the quantitation of ATP Release. Supplied as 2 µmole of lyophilized adenosine 5' triphosphate. 5 µL added to a test sample provides a 2 nmole standard.

CHRONO-LUME (P/N 395) – For the quantitation of ATP Release in the detection of aspirin use and the diagnosis of Storage Pool and Secretion Disorders. Luciferin-Luciferase binds with ATP, generating a light, which is proportional to the amount of ATP released by the platelets in the test sample.

NOTE: CHRONO-LUME reaction is time and temperature dependent. Be sure to incubate with each sample for two minutes only before starting test.

Collagen (P/N 385) – A lag phase follows addition of reagent to test sample, during which collagen polymerizes into fibrils for platelet activation. Low concentration collagen (1-2 µg/mL) is inhibited by cyclooxygenase inhibitors such as aspirin; normally, higher concentrations (5µg/mL) are not affected.

Epinephrine (P/N 393) – Shape change is not seen with this agonist. Higher concentrations (>5 µM) produce a biphasic curve with second-wave aggregation dependent on thromboxane A2 synthesis. Epinephrine is not recommended as a standard agonist for Whole Blood testing clinically, as fewer than 50% respond to this very weak agonist. The recommended anti-coagulant for Whole Blood testing with epinephrine is 1.5% trisodium citrate with 2 U Heparin per mL of citrate.

Ristocetin (P/N 396) – For the detection of von Willebrand Disease (a quantitative or qualitative defect in plasma von Willebrand Factor) and Bernard Soulier Syndrome (a lack of platelet membrane glycoprotein GPIb). Ristocetin results can be affected by aspirin and Glanzmann's Thrombasthenia with Aggregation-Disaggregation pattern.

Thrombin (P/N 386) – For the quantitation of maximum ATP Release at 1U/mL, not for aggregation. Secretion in response to Thrombin is independent of thromboxane synthesis. Absent or decreased secretion to Thrombin may be indicative of storage pool deficiency or a secretion defect.

Material Required But Not Provided

1. Aggregometer

2. Cuvettes

3. Stir Bars

4. Micropipettes - Adjustable from 0.5µL to 100µL required for reagents.

5. Pipettes - 100µL to 1 mL required for blood samples.

6. Sterile physiological saline for irrigation (0.85% w/v) for CHRONO-PAR^{R} Reagent preparation and for dilution of the Whole Blood specimen

Avoid blood bank saline because it may be an incorrect osmolarity. Cell counter diluents are not suitable because they contain EDTA, which inhibits platelet aggregation. Infusion salines are inappropriate because they contain benzyl alcohol (or other preservatives). Such preservatives inhibit platelet function.

7. Sterile bottled distilled water is suitable for CHRONO-PAR^{R} Reagent preparation.

Should be pyrogen free (ATP free) for reconstituting reagents and not contain preservatives such as benzyl alcohol which inhibits platelet function.

8. Ice for maintaining Reconstituted Working Reagents at appropriate temperatures.

9. 15 mL conical tubes

10. KimWipes®

INTERPRETATION OF RESULTS

Aggregation and luminescent ATP secretion curves in blood and PRP can be interpreted as follows:

- By direct comparison to a normal drug free control which also provides real time quality control.
- Comparison to published normal values that can be verified and reproduced by any laboratory.
- Collagen or Arachidonic Acid releases ATP equal to or greater than 50% of that released in response to Thrombin. ADP and epinephrine induce less ATP release.
- In a study of one hundred and six patients with storage pool deficiency (SPD), 23% had normal optical (PRP) aggregation responses to ADP, epinephrine and collagen; and 44% had miscellaneous aggregation abnormalities. The authors concluded that SPD is common, heterogeneous and not necessarily associated with optical (PRP) aggregation abnormalities.[7]
- Simultaneous measurement of aggregation and ATP release provides unequivocal evidence of dense granule secretion.[5] The threshold value at which storage pool deficiency should be considered has been reported to be less than 0.5 nmole ATP in response to 1U thrombin.[4]

LIMITATIONS

- Tests should be performed within 3 hours of venipuncture.
- Many drugs inhibit platelet function. Unless the aim of testing is to demonstrate drug-induced inhibition, patients should be drug free for two weeks prior to testing.
- Platelet count in test sample must be above 100,000 when testing in whole blood with ADP.
- Further Clinical and Laboratory evaluation may be required to confirm diagnosis.

QUALITY CONTROL

It is good laboratory practice to run a drug free normal control whenever reagents are reconstituted or thawed.

WARRANTY

CHRONO-PAR® AND CHRONO-LUME® REAGENTS which fail to demonstrate aggregation and release in drug-free normal controls before expiration and when stored and reconstituted as directed are replaced at no charge. This warranty applies only in the United States.

CONDUCTING TESTS

P/N	Description	Unit Volume	Stock Conc. WB/PRP	Final Conc. WB/PRP	Volume Per Test		Tests Per Unit	
					WB*	PRP**	WB	PRP
386	Thrombin	1.0 mL	10 Unit/mL	1 Unit	100µL	50µL	10	20
385	Collagen	1.0 mL	1 mg/mL	2µg/mL	2µL	1µL	500	1000
390	Arachidonic Acid	0.7 mL	50 mM	0.5 mM	10µL	5µL	70	140
396	Ristocetin	0.5 mL	125 mg/mL	1.0 mg/mL/ 1.25 mg/mL	8µL	5µL	62	100
395	Chrono-lume	4x1.25 mL	N/A	N/A	100µL	50µL	50	100
384	ADP	5.0 mL	1 mM	10µM	10µL	5µL	500	1000
393	Epinephrine	5.0 mL/50 mL	10 mM/ 1 mM	50µM/10µM	5µL	5µL	1000	10000
387	ATP Standard	5.0 mL	2µmole	2 nmole	5µL	5µL	1000	1000

* In a 1.0 mL sample – typically 450 µL blood diluted with 450µL of physiological saline plus 100 µL of CHRONO-LUME Reagent.

** In a 500µL sample – typically 450 µL platelet rich plasma plus 50 µL of CHRONO-LUME Reagent. (Reduce volumes by HALF with P/N 365 rubber spacers for a 250 µL microvolume sample.)

NOTE: Multiple Stock solutions are not required. To change Final Concentration, adjust pipette volume.

Since each test requires only micro-volumes of reagent, it is essential that introduction of excess reagent be avoided. Therefore, remove the excess reagent

adhering to the outside of the tip by wiping the outside of the micropipette tip after drawing the reagent.

It is important that the tip of the micropipette is immersed in the sample and the reagent forcefully injected. DO NOT introduce the reagent above the sample in the cuvette since the reagent will cling to the side of the cuvette and will not mix with the sample.

NOTE: For best results, reconstituted working reagents should be kept on ice. Reconstitued CHRONO-LUME®, EPINEPHRINE and ARACHIDONIC ACID should be kept on ice and in the dark.

PROCEDURES

PROCEDURES utilizing AGGRO/LINK® with Windows–compatible software and following NCCLS GP2-A3 format are available on disk or via Email (proced@chronolog.com) for:

- Whole Blood Aggregation
- Whole Blood Aggregation with ATP Release
- Optical Aggregation with PRP
- Optical Aggregation with ATP Release

CONTROL AND PATIENT SAMPLES- collect drug-free normal control and patient samples into sterile evacuated tube with a non-wettable lining and 1/10 volume of 3.2 or 3.8% buffered sodium citrate. Draw 20-30 mL blood for Optical PRP testing or 5-10 mL blood for Whole Blood testing.

Normal Ranges

NOTE: The following Normal Ranges were obtained from various laboratories and publications. They should be used as a guideline only. Normal ranges should be established for aggregation and ATP release in each and every laboratory.

Normal Ranges in Platelet Rich Plasma (Mean +/- 1 SD)			
Reagent	**Conc.**	**Agg. (%)[2]**	**ATP (nmole)**
Thrombin	1 Unit	N/A	>0.5 [3]
Collagen	2µg/mL	70 - 94	0.74 - 1.92[9]
Arachidonic Acid	0.5 mM	74 – 99[9**]	0.56 - 1.40[9]
ADP	5µM	69 - 88	0.41 - 0.63[2]
	10µM	71 - 88	0.5 – 1.06[9]
Epinephrine	5µM	78 - 88	0.40 - 0.52[2]
Ristocetin	1.25 mg/mL	87-102[9]	N/A

(** +/- 2 SD)

Normal Ranges in Whole Blood (Mean +/- 2 SD)			
Reagent	**Conc.**	**Agg. (ohms)[1]**	**ATP (nmole)[1]**
Thrombin	1 Unit	N/A	>0.5[4]
Collagen	2µg/mL	15 - 27	0.5 - 1.7
	5µg/mL	15 - 31	0.9 - 1.7
Arachidonic Acid	0.5 mM	5 - 17	0.6 - 1.4
ADP	5µM	1 - 17	0 - 0.7
	10µM[3]	6 - 24	0.38-1.71[13]
Ristocetin	1.0 mg/mL	> 5Ω; < 70 sec. lag time[4]	N/A

CALCULATION OF ATP RELEASE-the AGGRO/LINK Software calculates ATP release. If using a chart recorder, the following formula is used for calculation:

$$\frac{\text{Luminescence of test}}{\text{Gain of Test}} \times \frac{\text{Gain of standard}}{\text{Luminescence of standard}} \times 2 \text{ nmoles}$$

REFERENCES

(1) Ingerman-Wojenski CM and Silver MJ: A Quick Method for Screening Platelet Dysfunctions Using the Whole Blood Lumi-Aggregometer. Thromb Hemostas 51-2 (154-156) 1984.

(2) White M MC, Jennings LK: Platelet Protocols: Research and Clinical Laboratory Procedures. Academic Press, 1999.

(3) Podczasy JJ, Lee J, Vucenik I: Evaluation of Whole-Blood Lumiaggregation.Clin Appl. Thrombosis/Hemostasis, 3(3):190-195, 1997.

(4) Sweeney JD, Hoernig LA, Michnik A and Fitzpatrick JE: Whole Blood Aggregometry. Influence of Sample Collection and Delay in Study Performance on Test Results. Am J Clin Pathol 1989; 92:676-679.

(5) Feinman RD, Detwiler TC and Ingerman-Wojenski C: The Lumi-Aggregometer as a Research and Clinical Tool. The Plts.: Physiology and Pharmacology. Copyright 1985 by Academic Press, Inc.

(6) Sirridge, M: Laboratory Evaluation of the Bleeding Patient. Clinics in Laboratory Medicine, Vol. 4, No. 2, June 1984.

(7) Nieuwenhuis HK, Akkerman J-W N and Sixma JJ: Patients With a Prolonged Bleeding Time and Normal Aggregation Tests May Have Storage Pool Deficiency: Studies on One Hundred Six Patients. Blood 70-3 (620-623) 1987.

(8) Sweeney JD, Hoernig LA and Fitzpatrick JE: Whole Blood Aggregation in von Willebrand Disease. Am J Hematol 32: 190-193 (1989).

(9) UMDNJ, Spec. Hemostasis Lab, New Brunswick, NJ.

(10) Sweeney JD, Hoernig LA, Behrens AN, Novak E, and Swank RT: Von Willebrand's Variant (type II Buffalo). American Journal of Clinical Pathology Vol. 93, No. 4, (522-525) 1990.

(11) Miller JL: Platelet-Type von Willebrand's Disease. Thrombosis and Hemostasis Vol 7, No. 4, 1985.

(12)Taylor ML, Misso NLA,Stewart GA,Thompson PJ: The effects of varying doses of aspirin on human platelet activation induced by PFA, Collagen and arachidonic acid. BJ J Clin Pharmac., (1992), 33, 25-31.

(13) Ingham Medical Center, Lansing, MI.

(14) Riess H, Braun G, Brehm G and Hiller E: Critical evaluation of platelet aggregation in human whole blood. Am J Clin. Pathol. Vol. 85 No. 1 (50-56) 1986.

EXPECTED RESULTS

AGGREGATION RESPONSE WITH SELECTED ABNORMALITIES					
Reagent	**Final Concentration**	**Aspirin Effect**	**Von Willebrand & Bernard Soulier**	**Storage Pool/ Secretion Defect**	**Glanzmann's Thrombasthenia**
ADP	5 – 20 µM	**N, R ***	**N**	**N, R** *	**A**
Arachidonic Acid	0.5 mM	**A**	**N**	**N**	**A**
Collagen	2 – 5 µg/mL	2µg/mL: **R**; 5 µg/mL: **N**	**N**	**N**	**A**
Epinephrine	10 – 50 µM	**R***	**N**	**R** *	**A**
Ristocetin	0.25 – 1.5 mg/mL	Qualitative[6] Defect	** **A,R,H** *** >70 sec. Lag (vW)	**N**	**Qualitative Defect[14]**

* Second-wave Inhibited
** Type 2B and Platelet-type von Willebrand increased at low concentration 0.2 -0.6 mg/mL[10, 11]
*** To distinguish between von Willebrand & Bernard Soulier, add normal plasma or cyoprecipitate to patient sample, vW patient will respond, Bernard S oulier will not.[11]

ATP SECRETION WITH SELECTED ABNORMALITIES					
Reagent	**Final Concentration**	**Aspirin Effect**	**Von Willebrand & Bernard Soulier**	**Storage Pool/ Secretion Defect***	**Glanzmann's Thrombasthenia**
ADP	5 - 20 µM	**A, R**	**N**	**A,R**	**A**
Arachidonic Acid	0.5 mM	**A**	**N**	**A,R**	**R**
Collagen	2 – 5 µg/mL	**R**	**N**	**A,R**	**R**
Epinephrine	10 – 50µM	**A**	**N**	**A,R**	**A**
Ristocetin	0.25 – 1.5 mg/mL	**--**	**--**	**--**	**--**
Thrombin	1 Unit	**N**	**N**	**A,R**	**R[5]**

* Higher concentrations of any agonist including Thrombin up to 5 Units will induce ATP secretion with a Secretion disorder but <u>will not</u> with a Storage Pool Defect.[5]

Key: A – Absent **H** – Hyper **N** – Normal **R** - Reduced
(Compared to Normal Range)