UPMC
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Procedure Name: LPS ACTIVATION OF MAPK SIGNALING IN WHOLE BLOOD SAMPLES

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Authors: P. Grom and T.V.Shankey

Supersedes Procedure: NA

Distribution:

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Laboratory Supervisor_____**Date**_____

Technologist Annual Review

Date	Signature

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LPS ACTIVATION OF MAPK SIGNALING IN WHOLE BLOOD SAMPLES

Principle

LPS (lipopolysaccharide) is known to activate multiple signaling pathways in peripheral blood monocytes via the Toll-Like Receptor 4 (TLR4) complex. Following LPS exposure, monocytes rapidly activate all three major MAP Kinase pathways – ERK, P38, and SAP/JNK, in addition to PI3 Kinase and IKK/NF□B pathways. Flow cytometry provides a unique methodology to monitor these signaling pathways, allowing a coordinated measurement of multiple phospho-epitopes in the context of cell surface plus other cellular markers.

This same approach can be used to study the impact of specific signaling pathway inhibitors to determine which downstream signaling pathways are affected. Overall, monitoring signal transduction pathways in stimulated whole blood (and other similar types of samples) offers a unique way to test and validate antibodies, specific agonists, or antagonists using a relevant biological system. In addition, this approach can be used to monitor the activity of targeted therapies (inhibitors) in vivo, or to monitor the prior inhibition of monocyte signaling due to LPS exposure.

In AML (Acute Myelogenous Leukemia) patients, this strategy has been used to study the impact of several targeted agents on the MAPK and PI3K pathways in addition to monitoring phosphorylation of the ribosomal S6 protein. This approach provides unique insight into the biologic heterogeneity of human leukemias and offers the potential to monitor individual patients' responses to targeted agents, including the potential for "real-time" drug monitoring.

Specimen or Product Requirement

Whole blood collected into K2EDTA or sodium heparin; 4.0-5.0 mls; stored at room temperature until tested

Volume is not critical. Any volume may be submitted, however, limited testing may result.

Sample Suitability:

a) Sample testing should (ideally) begin within 1-4 hours of collection. The blood sample must be used as soon as possible in order to preserve appropriate signaling capabilities.

b) Whole blood samples which display gross hemolysis are unacceptable.
c) The impact of other anticoagulants (e.g. ACD) has not been tested.

Reagents and Supplies

Supplies	**Supplier**	**Catalog number**
LPS (liposaccharides); dilute to 50 ug/ml in 1XPBS; store this working dilution at 40 C; stable for up to 6 months	Sigma	catalog #L4516 – from E. coli 0127 :B8
Surfact-Amps® X-100 (Triton X-100), 10% aqueous solution ; prepare working solution by diluting 116 ul stock with 10 mls 1XPBS; store stock and working solution at room temperature; working dilution is stable for 1 month	Pierce	28314
Formaldehyde, 10%(methanol-free); room temperature in the dark; use within 6 months of opening	Polysciences, Inc	04018
Fetal Bovine Serum, store at 4^{o} C	PAA Laboratories, Inc	A15-701
Phosphate Buffered Saline (PBS); calcium- and magnesium-free;	Gibco	20012
Methanol, 100%, reagent grade; dilute to 50% with 1XPBS; store aliquots at -200 C; use at 4^{o} C	Sigma-Aldrich (or equivalent)	179337
Wash Buffer – 4% Fetal Bovine Serum (FBS) in 1XPBS; filtered through 0.22 micron sterile filter; 500 ml		
Deionized water		
CD14-PC7 (clone RMO52),.;	Beckman Coulter, Inc	A22331
P-p38 MAPK-Alexa488	Cell Signaling	4551

[T180/Y182]; (clone 28B10);	Technology®;	
P-SAPK/JNK-PE [T183/Y185]; (clone 69) (custom conjugate prepared by Custom Design Service, Advanced Technology, Beckman Coulter, Inc.)	Cell Signaling Technology®	9255
P-ERK-Alexa647 [T202/Y204]; also known as P-p44/42 MAPK; (clone E10)	Beckman Coulter, Inc.	A24062
P-S6-Pacific Blue; [S235/236]; (clone D57.2.2E) (custom conjugate prepared by Custom Design Service, Advanced Technology, Beckman Coulter, Inc.)	Cell Signaling Technology®	4858
12 X 75 mm polypropylene tubes	VWR	catalog numbers depend on colors desired
Tube racks; for 12 X 75 mm tubes, for 50 ml tubes, and for antibody dilutions		
50 ml conical tubes; sterile, polypropylene	VWR	20171-028
Pipet Tips appropriate for pipettors used		
Disposable serological pipettes; 1 ml, 5 ml, 10 ml, 25 ml		
Biohazardous Waste Receptacle		
Personal Protective Equipment ; gloves, labcoat, safety shield, protective covering for countertop, etc.		
Cotton-tipped applicators – 6-inch		
Aluminum Foil		
Vacuum Filter System, 0.22 microns ; 500 ml	VWR(or equivalent)	87006-064
Ice and ice bucket		

Instrumentation

Refrigerator @ 2-6o C	
Freezer @-200C	
Automatic Pipettor	
Pipettors to deliver from 1 to 1000 ul	(e.g. Rainin or Pipetman)
Stopwatch	
Timer	
Circulating 37^{o} C water bath	
Vortex	
Centrifuge, variable speed	(e.g. Beckman-Coulter Allegra 6R)
Flow Cytometer	[e.g. Gallios™ (3-lasers/10 colors) or FC500™ (2 lasers/5 colors)]
Waste Vacuum, optional Centrifuge	

Procedure

Safety and Cautionary Notes

Biologic hazard

All blood samples may potentially harbor infectious agents. Universal precautions must be used in all steps of this procedure involving blood or its derivatives.

Formaldehyde is a suspected carcinogen. It is an irritant and a sensitizer and is highly toxic. Use in well ventilated areas. Wear gloves.

Methanol is considered a poison. If swallowed, it may be fatal or cause blindness. It is harmful if inhaled or absorbed through the skin. It is also highly flammable and can cause irritation to the eyes, skin, and respiratory tract. Target organs = kidneys, heart, CNS, liver, eyes

LPS is a pyrogen. It may cause fever. Do not use if skin is cut or scratched. Always wear gloves when using.

Triton X-100 is an irritant. It causes respiratory tract, eye, and skin irritation. It may be harmful if swallowed. It contains material which causes damage to mucous membranes, upper respiratory tract, skin, and eyes

Acquisition

For acquisition using a Gallios™ flow cytometer -- Navios™ software, revision b9.2 or greater

For acquisition using an FC500™ flow cytometer – CXP version 2.2 or greater

Procedure

Before starting this procedure, prepare an experiment worksheet to aid in the critical timing steps. (See attachment 15.1) The experimental procedure below is limited to one time point (10 minutes). For experiments adding specific signaling pathway inhibitors (not outlined here), whole blood sample(s) is incubated at 37o C with inhibitor(s) for an appropriate time (depending on the specific inhibitor) before the addition of LPS. For further details, refer to the Special Notes section.

Label 12 X 75 mm test tubes for the previously designed experiment. There will be 5 control tubes and as many experiment tubes as needed. [controls = One CD14-only tube (no LPS and no phospho-antibodies), two unstimulated tubes – RT and 37o C tubes without LPS but containing all of the phospho-antibodies and CD14, and two LPS-stimulated tubes containing CD14 PC7 plus either P-p38 Alexa 488 or P-SAPK-PE as compensation controls]

Remove the 50% methanol from the freezer and place it into an ice bath for use at a later point in the procedure.

Pipet 100 ul of blood sample into the bottom of each appropriately labeled tube. Use a cotton-tipped applicator to remove any blood from the side of the tube. (see Limitations section)

Add 100ng LPS (2ul of working dilution) to the first of the designated stimulation tubes. Place that tube into the water bath and start a stopwatch. At the appropriate time interval, add LPS to the next tube and place it into the water bath. Continue for all tubes in the stimulation part of the experiment. Refer to Attachment 15.1.

Continue to use the staggered start to place the 37o C no LPS control tube and the CD14-only tube into the water bath. Incubate the RT control at room temperature. Refer to Attachment 15.1.

At the 10-minute mark, remove the first tube in the timed sequence from the water bath and add 65ul of 10% formaldehyde to the tube. Immediately vortex and place it into a tube rack. Continue adding 65ul of formaldehyde to each tube in the timed sequence, vortexing between each one. NOTE: THIS IS A CRITICAL STEP. FORMALDEHYDE STOPS THE LPS ACTIVATION AND FIXES THE CELL.

Incubate each tube for a total of 10 minutes at room temperature.

After exactly 10-mintes of incubation in formaldehyde, pipet 1 ml of Triton solution into each tube. NOTE: DO NOT MANIPULATE THE TUBES IN ANY WAY. ADD TRITON SOLUTION DIRECTLY TO THE TREATED WHOLE BLOOD/FORMALDEHYDE MIX. Vortex each tube. Place all tubes into the 37o C bath for 15 minutes.

Remove tubes from the water bath and vortex them again. Add 2 ml of cold (4o C) wash buffer (4% FBS/PBS) to each tube.

Centrifuge all tubes at 500 X G for 4 minutes.

Remove as much of the supernatant as possible exercising care to preserve the cell pellet.

Place the first tube on the vortex and add 1 ml of cold (4o C) 50% methanol. Place the tube into an ice bath. Repeat for all tubes in the test system.

Incubate all tubes on ice for 10 minutes.

At the end of the incubation, centrifuge all tubes at 500 X G for 4 minutes.

Remove as much of the supernatant as possible exercising care to preserve the cell pellet.

Pipet 2 ml of cold (4o C) wash buffer (4% FBS/PBS) to each tube.

Centrifuge all tubes at 500 X G for 4 minutes.

Remove as much of the supernatant as possible exercising care to preserve the cell pellet.

Add antibodies (concentrations and volumes must have been previously defined) and cold wash buffer to a final volume of 100 ul. (It would be best to prepare a cocktail of desired antibodies. Pre-dilute all antibodies in order to add a total volume of 100 ul to each tube. This ensures that the antibody concentration for each tube is “identical”.)

Incubate all tubes at room temperature for 30 minutes in the dark.

At the end of the incubation, add 2 ml of cold (4o C) wash buffer (4% FBS/PBS) to each tube.

Centrifuge all tubes at 500 X G for 4 minutes.

Remove as much of the supernatant as possible exercising care to preserve the cell pellet.

Resuspend the cells in 500 ul of wash buffer. Vortex. Analyze on the flow cytometer.

Special Notes

Since the FC500™ does not have a 405 nm (violet) laser, antibody conjugates containing Pacific Blue cannot be used. For experiments using Pacific Blue (e.g. P-S6 Pacific Blue), a Gallios™ flow cytometer equipped with a violet laser (or comparable instrument) must be ued.

Prepare wash buffer (4% FBS + PBS) ahead of time and keep stored in refrigerator at 4o C. Use a vacuum filtration system (container-topped filter) that is comprised of a 0.22 micron filter to remove particulates before utilizing the buffer in the test procedure.

Prepare 50% methanol ahead of time and keep stored at -20o C. Remove to an ice bath prior to use in the test procedure so that it more closely approximates 4o C in the test system.

All antibodies must be titrated for optimal results. Specific phospho-epitopes are maximally expressed at different time points after activation of a signaling pathway by a specific agonist. In many cases, the peak phospho-epitope signal will decrease significantly after the peak activation.

Limitations of the procedure

Failure to remove all blood from the side of the tube at the very beginning of the procedure will result in contamination by unfixed cells.

Inappropriate incubation times with LPS for the designated phospho-antibodies may yield incorrect or missing results within the signaling pathways.

Each phospho-epitope has its own time course, and one timepoint may not be optimal for another signaling pathway.

Result Reporting

Record results on appropriate worksheet. This procedure is for research use only. All results must be interpreted only by knowledgeable personnel.

Acceptable Endpoints

ANY?

Quality Control

Flow Cytometer Daily Quality Control

Quality Control Data

Instrument QC is printed and kept in the QC notebook at the flow cytometer.

Status Page from the experiment – includes all voltage levels, discriminator values, and any compensation information

Internal test controls

One CD14-PC7 only tube (no LPS and no phospho-antibodies). This is used as a compensation control for spectral overlap of PC7 into the PE channel.

Two unstimulated control tubes – RT (room temperature) and 37o C tubeswithout LPS but containing all of the phospho-antibodies and CD14-PC7

Two LPS-stimulated control tubes (37o C); one containing CD14-PC7 plus P-SAPK-PE as compensation control for Alexa 488 versus PE; one containing CD14-PC7 plus P-p38-Alexa 488 as compensation control for PE versus Alexa 488.

References

This procedure developed by P. Grom and T.V. Shankey.

a) Jacobberger JW, Sramkoski RM, Frisa PS, Ye PP, Gottlieb MA, Hedley DW, Shankey TV, Smith BL, Paniagua M, and Goolsby CL. Immunoreactivity of STAT5 phosphorylated on tyrosine 694 as a cell-based measure of Bcr/Abl kinase activity. Cytometry 54A: 75-88, 2003.

b) Chow S, Hedley D, Grom P, Magarri R, Jacobberger J, and Shankey TV. A whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of phospho-epitope expression in leukocyte subpopulations. Cytometry 67A: 4-17, 2005.

c) Shankey TV, Forman M, Scibelli P, Cobb J, Smith CM, Mills R, Bernal-Hoyos E, Van Der Heiden M, Popma J, Keeney M. An optimized whole blood method for flow cytometric measurement of Zap-70 protein expression in Chronic Lymphocytic Leukemia. Cytometry 70B: 259-269, 2006.

d) Hedley DW, Chow S, Goolsby C, and Shankey TV. Pharmacodynamic Monitoring of Molecular-Targeted Agents in the Peripheral Blood of Leukemic Patients Using Flow Cytometry. Toxicol Pathol. 36: 133-139, 2008

Appendices

Revision	Change	Rationale	Standards	Start Date	End Date
0	Creation				
1					
2					
3					