ABEL®
CELL ACTIVATION TEST KITS WITH PHOLASIN®

from

Knight Scientific Limited

ABEL® CELL ACTIVATION TEST KITS WITH PHOLASIN®

for monitoring the production of free radicals and other reactive species

The ABEL®[1] (Analysis By Emitted Light) cell activation test kits for whole blood and isolated cells probe for the production by leucocytes of free radicals and other reactive species. This occurs during the activation of the superoxide-generating NADPH oxidase system, the so-called respiratory burst. The tests may also be used to monitor superoxide production by other types of cells that contain the NADPH oxidase system (e.g. fibroblasts, chondrocytes, mesanglial cells) as well as the production of superoxide by enzyme systems such as xanthine/xanthine oxidase. Additionally, the tests can be used to monitor degranulation in leucocytes and to identify cells that are prone to degranulate.

These unique chemiluminescent assays require only 1μL of whole blood or a few thousand isolated cells and are complete well within 10 minutes.

The kits contain the unique light-emitting protein Pholasin® as well as the cell stimulants PMA (phorbol-myristate-acetate) and fMLP (formyl-methionyl-leucyl-phenylalanine) together with all necessary buffers for reconstituting the reagents and performing the tests. The ABEL® Whole Blood Test Kits also contain blood dilution buffer and tubes in which to carry out the dilution, as well as an adjuvant that enhances the chemiluminescent signal in the presence of red blood cells and other blood constituents.

Cell Activation Test Kits with Pholasin® are easy-to-use, rapid, ultra-sensitive (can be used with capillary blood) chemiluminescent tests that measure real-time production of free radicals and degranulation .

They can be used to:

- measure the respiratory burst in leucocytes
- study abnormalities in NADPH oxidase such as occur in different types of chronic granulomatous disease (CGD) and various polymorphisms
- distinguish between receptor activation and intracellular activation of NADPH oxidase
- monitor changes in the activation of the NADPH oxidase in response to complement activation, infection, inflammation and medical intervention, thus having applications in:
    - biocompatibility monitoring
    - drug evaluation and testing
    - QC of vaccines
    - surgery
    - disease management
    - assessing neutrophil activity following chemotherapy, transplantation etc.
    -
- monitor degranulation of myeloperoxidase
- ultra-sensitive detection of free radicals and oxidants produced by a range of cell types including brain cells.

[1] ABEL and Pholasin are registered trade marks of Knight Scientific Limited (KSL)

The NADPH oxidase when activated forms a complex of proteins that assembles at the inner surface of the plasma membrane. There a constant stream of superoxide is released to the outside of the cells via a continual transfer of electrons (from NADPH) through the membrane to oxygen. The term respiratory burst was used to describe this event because of the very large amounts of oxygen (10-20 times above normal) that are consumed during the oxidation of glucose to NADPH, which ironically is not via mitochondrial respiration. The membrane component of the NADPH oxidase also occurs on secondary granule membranes which fuse with other granules as well as the plasma membrane during deganulation. Degranulation frequently follows the activation of the oxidase depending upon the nature of the stimulus. And because the overwhelming amount of free radicals, oxidants and enzymes are released to the outside of the cells, methods that only measure intracellular hydrogen peroxide from the dismutation of superoxide quantify only a small part of the respiratory burst.

WHAT IS PHOLASIN® AND WHAT DOES IT DO?

Pholasin® is the photoprotein of the marine, rock-boring bioluminescent mollusc, *Pholas dactylus*, the Common Piddock. Pholasin® does not glow on its own[1] but needs to be switched on. Free radicals and other reactive oxygen species produced by activated leucocytes (and other cell types) turn on the light. Pholasin® is an ultrasensitive detector of activated leucocytes.

Pholasin® emits light in the presence of:

- free radicals: superoxide anion, singlet oxygen, hydroxyl radical and/or ferryl radical.
- oxidants such as: hypochlorous and hypobromous acids, chloramines, bromamines and peroxynitrite.
- enzymes: peroxidases and certain oxidases.

Pholasin® is isolated and purified by Knight Scientific Limited (KSL) from piddocks cultivated in land-based systems.

CLINICAL INTEREST

Various types of leucocytes respond in different ways to the presence of foreign bacteria and viruses that may at times enter the body. One important response is the activation of the enzyme system responsible for the production of superoxide anion (which is converted to hydrogen peroxide). The cells may also release enzymes normally contained within granules that produce reactive oxygen species such as hypochlorous and hypobromous acids.

Leucocytes also remove dead cells, in the heart for example, following a heart attack. These activities are important in aiding recovery. In fact, without these cells the body would be unlikely to survive even minor infections. However, during their work, the cells accumulate in vast numbers where they release damaging reactive oxygen species (ROS) and granule enzymes to the surrounding tissue and cause inflammation. To a small extent, inflammation can aid wound healing but, in general, inflammation is damaging and when it gets out of control it can lead to destruction of one organ after another, eventually killing the individual.

[1] In solution Pholasin® emits low-level light known as the resting glow

There are many diseases and conditions in which free radicals, reactive oxygen and nitrogen species play an important role. Leucocytes, which are major producers of these reactive species, are frequently implicated in the pathology and progress of these diseases.

WHAT DO LEUCOCYTES DO?

- They may become activated directly and produce superoxide, some of which is likely to be converted to hydrogen peroxide.
- They may degranulate[1] and release a range of enzymes.
- They may become primed by other substances (cytokines, chemokines, complement, viruses, bacteria, toxins, foreign materials) to respond later to stimuli in an enhanced (or depressed) way..
- They may leave the circulation and migrate to sites of inflammation where they become further activated to produce free radicals and to degranulate.
- Their proteolytic enzymes may destroy tissue, for example in the lungs and kidneys.
- The primary granule enzyme, myeloperoxidase is involved:
  - by producing hypochlorous acid and the longer-lived chloramines which can damage tissue such as cartilage.
  - by inactivating the anti-proteolytic enzymes which under normal circumstances prevent proteases such as elastase (another granule enzyme) from destroying tissue.

WHAT DO THE ABEL® TESTS DO?

- They monitor the kinetics of the activation of the NADPH oxidase system.
- They monitor the production and extracellular release of superoxide by cells.
- They identify if cells have been activated.
- They identify cells that may have been primed.
- They give a preview of the way the cells, circulating in the peripheral blood, are likely to respond at a site of inflammation.
- They are useful in identifying abnormalities in the leucocytes of so-called 'normal' control subjects.
- They may be useful in identifying patients at greater risk of complications to:
  - surgical procedures.
  - exposure to materials in medical devices such as those used in renal dialysis and cardiopulmonary bypass.
- They are useful in monitoring the progress of an inflammatory disease or complication and the response of a patient to drug intervention.

HOW DO THE TESTS WORK?

The tests work by stimulating the NADPH oxidase system[2] of cells such as neutrophils, eosinophils, monocytes and macrophages, in the presence of Pholasin®, either isolated or in diluted whole blood, and monitoring the resultant luminescent response. The two stimulants that activate the respiratory burst and that are provided with the kits are: the receptor stimulant fMLP and the phorbol ester PMA which enters the cell and activates

[1] Degranulation involves the fusion of the granule membrane with the plasma membrane and/or the membranes of other granules and the release of enzymes/proteins such as myeloperoxidase, elastase, and lactoferrin. These enzymes/proteins may for a while remain attached by receptors to the outside surface of the cell.

[2] Activation accompanies a 2-20 fold increase in non-mitochondrial consumption of oxygen (the so-called *respiratory burst*) and a continuous supply of NADPH generated via the hexose monophosphate shunt.

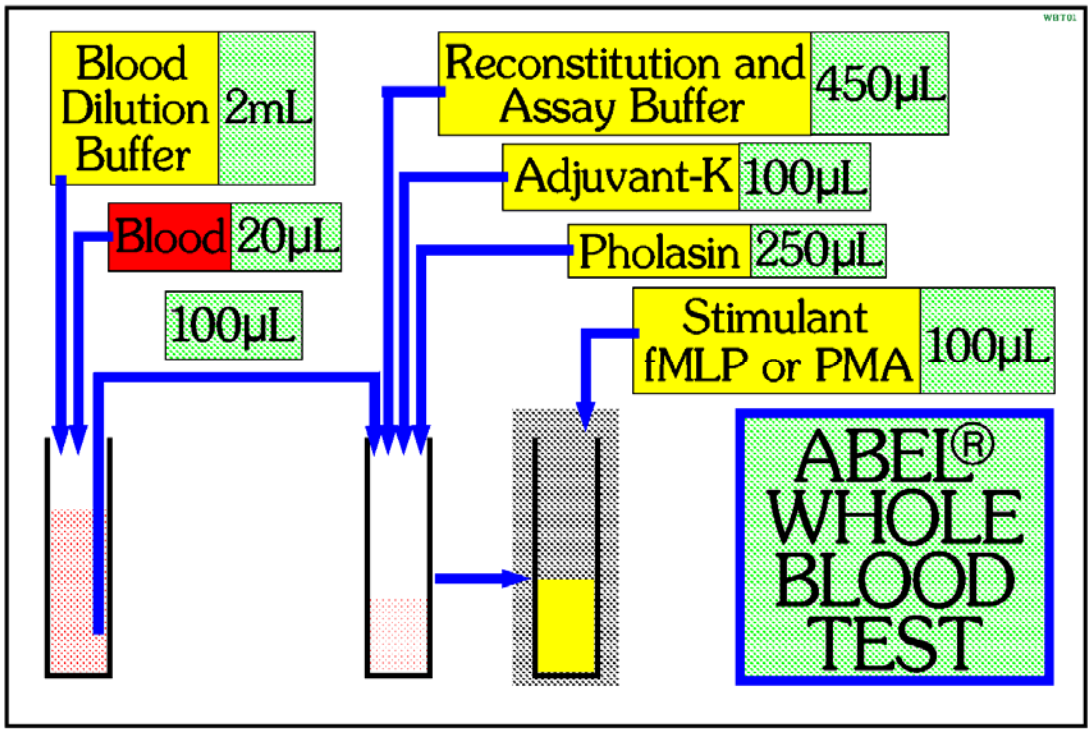
protein kinase C. Presentation of fMLP and PMA together enables the activation of the NADPH oxidase on the cell surface to be monitored simultaneously with the activation of the NADPH oxidase on the membrane of the secondary granules. PMA in fact activates the NADPH oxidase throughout the cell, but at a slower rate than fMLP; it also promotes degranulation.

While the test kits provide fMLP and PMA, other mediators such as platelet activating factor (PAF), anti-Fc receptor antibodies and lipopolysaccharides (LPS) may also be used. A low concentration of some of these mediators may cause the cells to become primed, perhaps by an upregulation of receptors, so that they subsequently respond in an enhanced way to further stimulation.

PROCEDURE WITH WHOLE BLOOD: TUBE LUMINOMETER

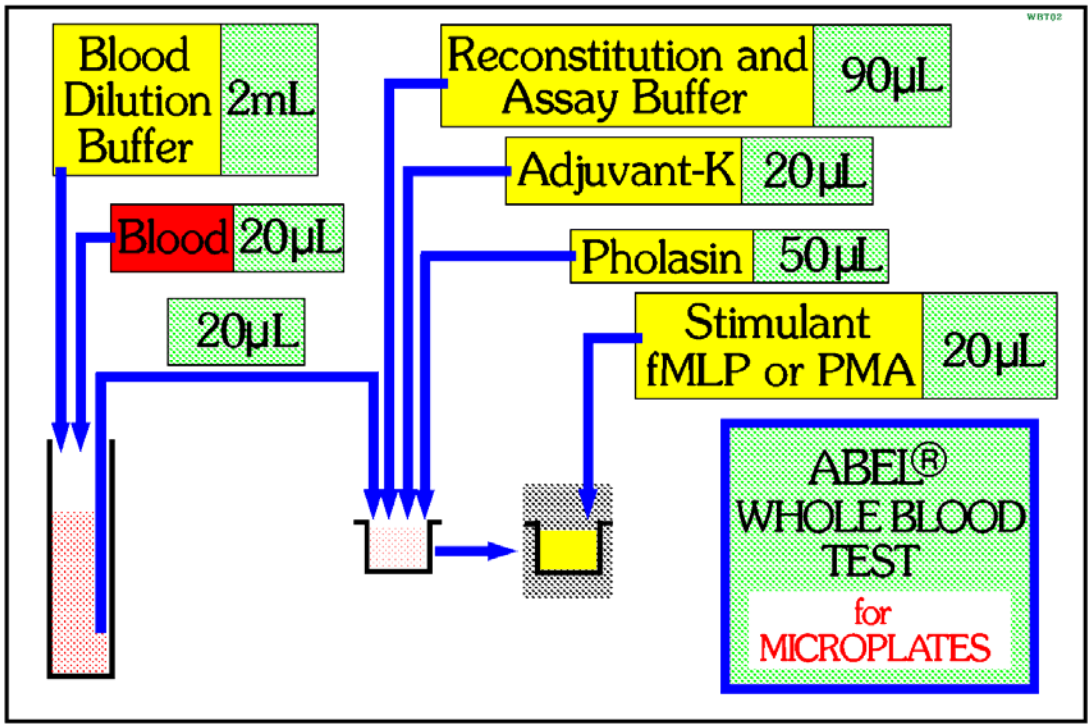
- 1:100 dilutions are made in 2mL of blood dilution buffer[1]; 100µL of diluted blood is used in each test luminometer cuvette.
- The cuvettes contain: 100µL diluted blood, 450µL assay buffer, 250µL Pholasin® (2.5µg), 100µL Adjuvant-K™ (1 enhancement unit).
- Note that Pholasin® may be added before or after the cuvette enters the luminometer.
- The mixture is incubated for 1 minute at 37°C.
- Light is then measured every second for 60 seconds. (The light readings are the accumulated light detected over each second and expressed as relative light units or mV in some luminometers).
- After 1 minute the stimulants are added.
- Light is measured for a further 5 minutes with fMLP and 8-10 minutes when PMA or a mixture of fMLP and PMA is used.

[1] The equivalent of 1µL of blood is used in the test . Because of the possible imprecision of measuring 1µL it is recommended that 20µL of blood is diluted with 2mL of blood dilution buffer and 100µL (containing 1µL of whole blood) is pipetted into the cuvette. However, if insufficient blood is available then a 1:100 dilution can be made with lower than 20µL; 1µL of whole blood can also be delivered directly to the cuvette.



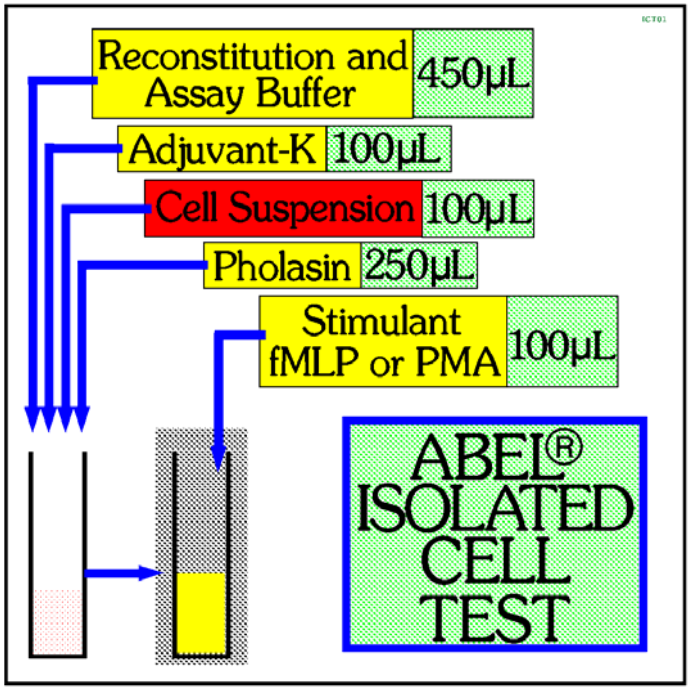
PROCEDURE WITH WHOLE BLOOD: MICROPLATE LUMINOMETER

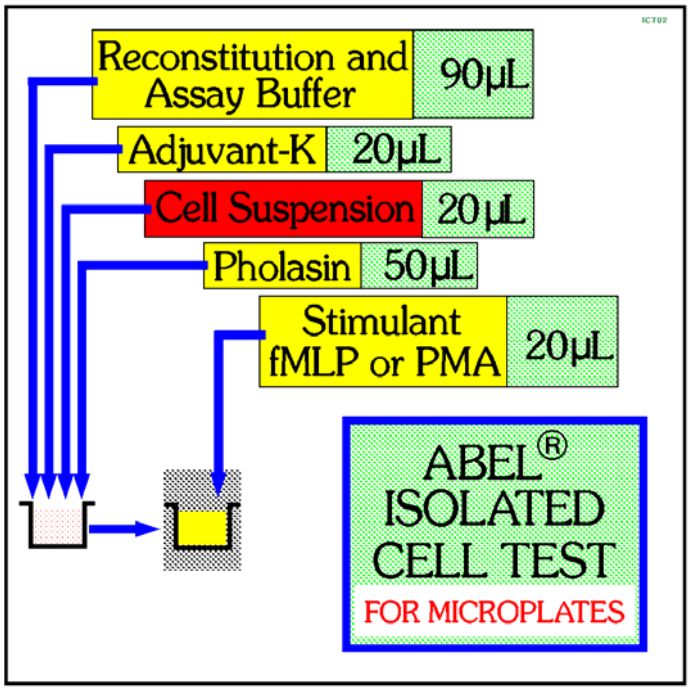
Follow instructions above but use volumes of reagents described in diagram below.



PROCEDURE WITH ISOLATED CELLS

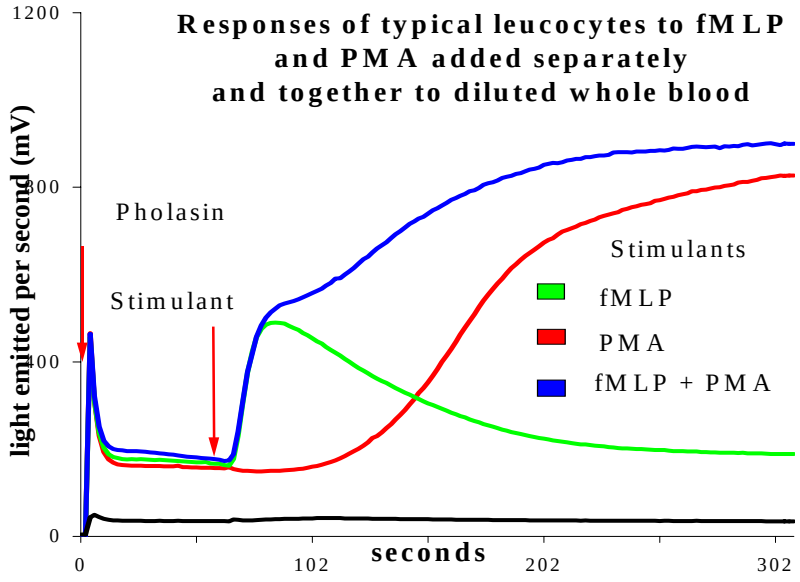
- Isolated cells from blood, lavage fluid, synovial fluid, or tissue culture for example, are transferred to reconstitution and assay buffer[1] and then 100µL (tube luminometer) or 20µL (microplate luminometer) of cell suspension is used in each test.
- The kit contains Adjuvant-K and the test can be carried out with or without Adjuvant-K™ depending upon the cell type and number of cells present. A trial with and without Adjuvant-K™ should be carried out first.
- All other procedures are identical to the procedure with whole blood.



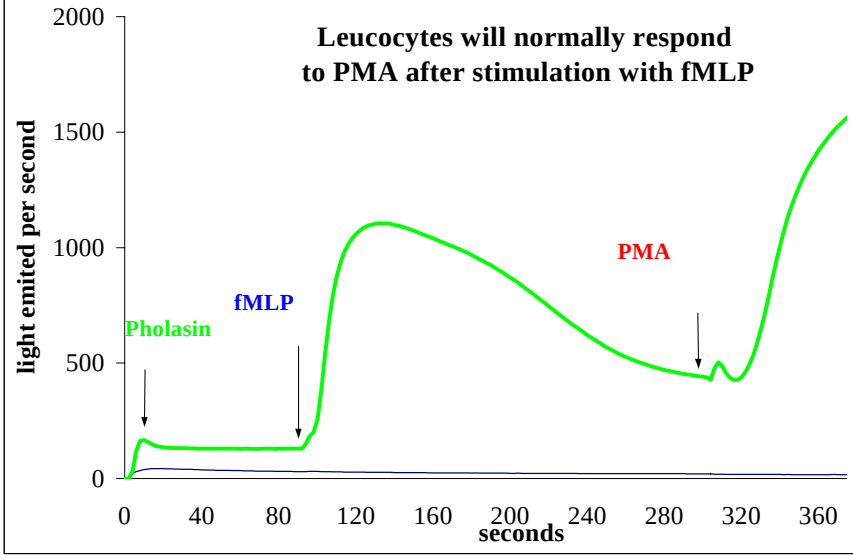


[1] Tissue culture media and physiological fluids may contain antioxidants that could quench free radicals produced when the cells are activated.

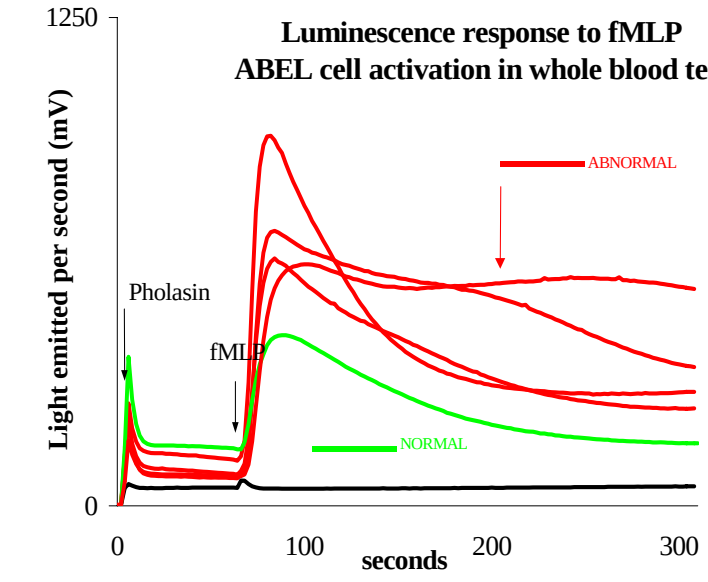
TYPICAL ANALYSIS



Luminescence response curve: 1µL whole blood in 1mL total volume Bio-Orbit 1251 luminometer
Each measurement is the amount of light detected over 1 second every second,
37°C continuous mixing
Composite graph showing response to receptor stimulant fMLP, intracellular stimulant PMA that acts directly on protein kinase C and a mixture of fMLP and PMA.

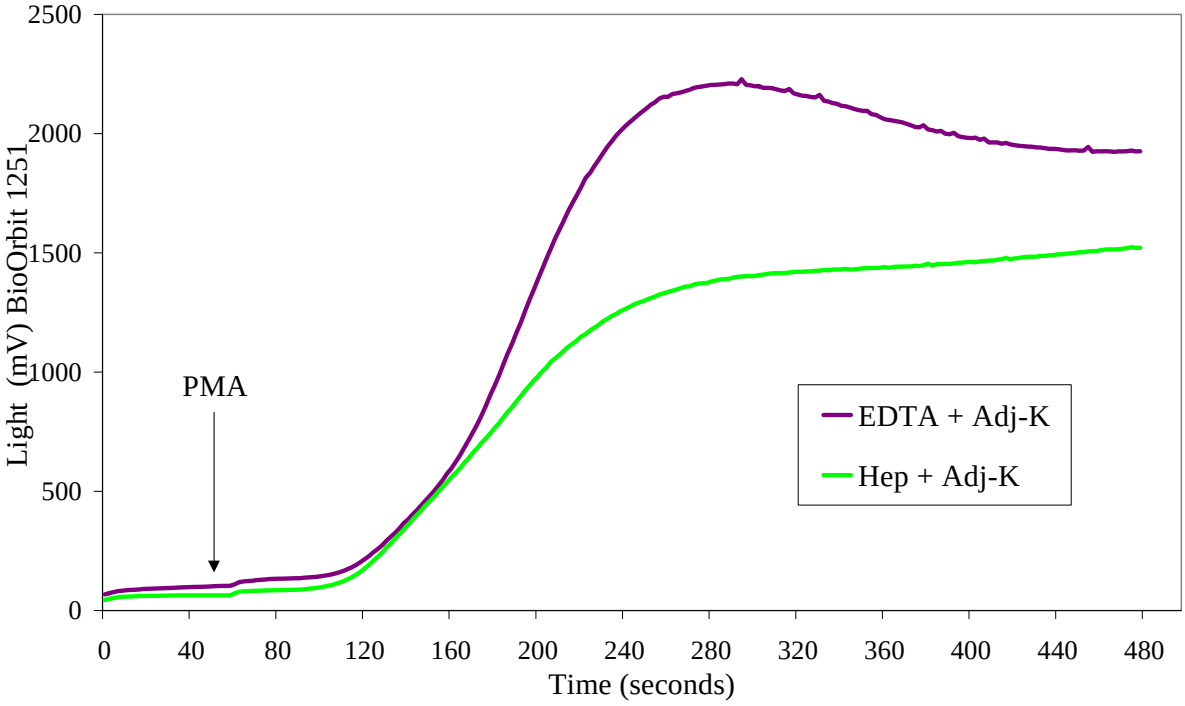


Neutrophils stimulated by the receptor stimulant fMLP can still respond to PMA; the converse (not shown) does not occur. This is because the fMLP works via receptors and acts (in most cases) solely on the NAPDH oxidase system of the plasma membrane whereas PMA activation of protein kinase C leads to the activation of the NADPH oxidase on both the plasma membrane and secondary granules.

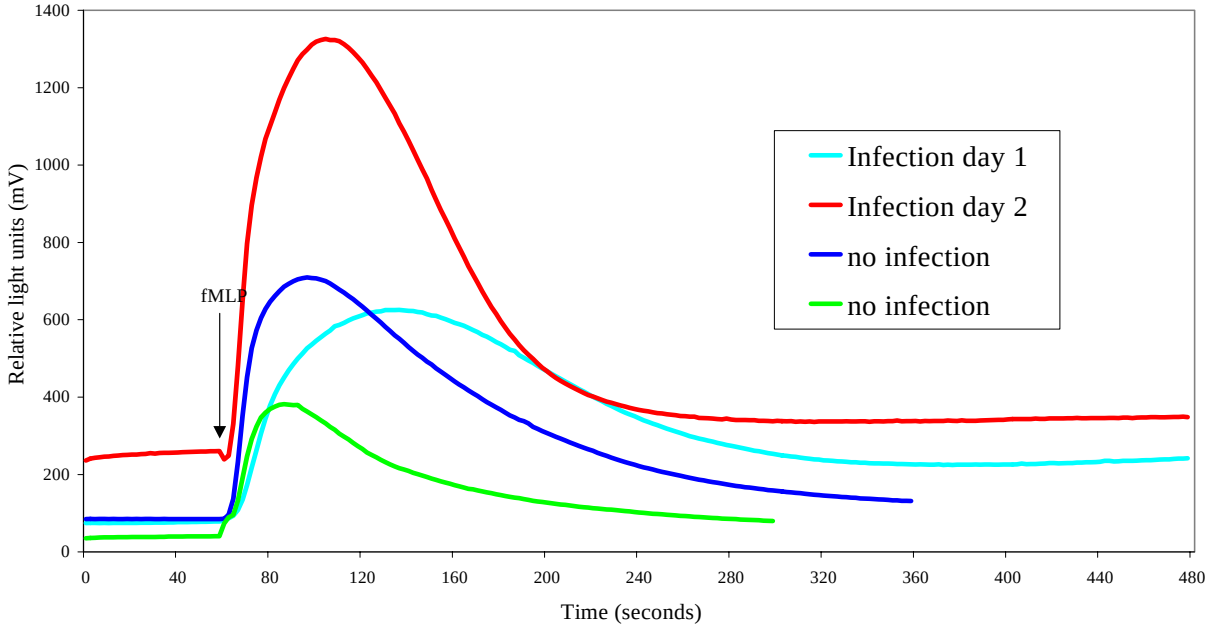


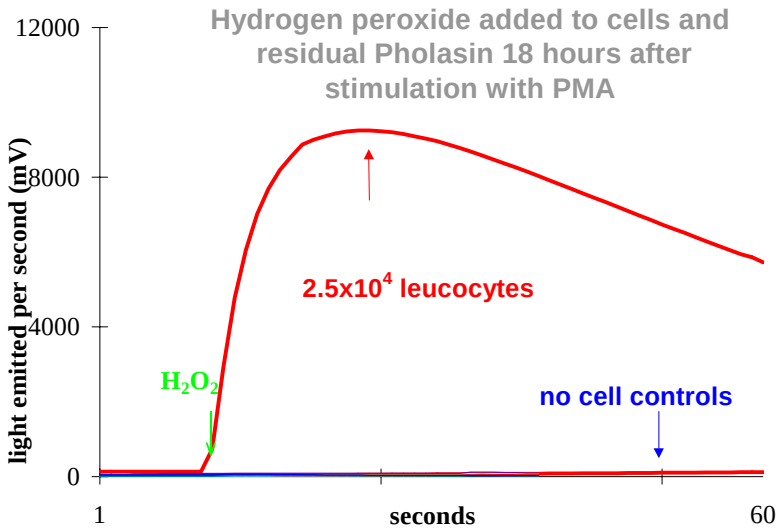
The response to fMLP shows some variation amongst individuals. A typical response to fMLP starts within 2-5s, reaches a peak in < 90s and tails off over about 2min. A variety of shapes in the response curves have been discovered in which the response to fMLP starts to approximate that normally seen when cells are stimulated with fMLP and PMA together. The most abnormal response (see arrow) occurred in a patient on dialysis 17 years. Anecdotal evidence links these abnormal shapes to patients with certain allergies and certain diseases.

ABEL® Whole Blood Test with Adjuvant-K™: Heparin vs EDTA

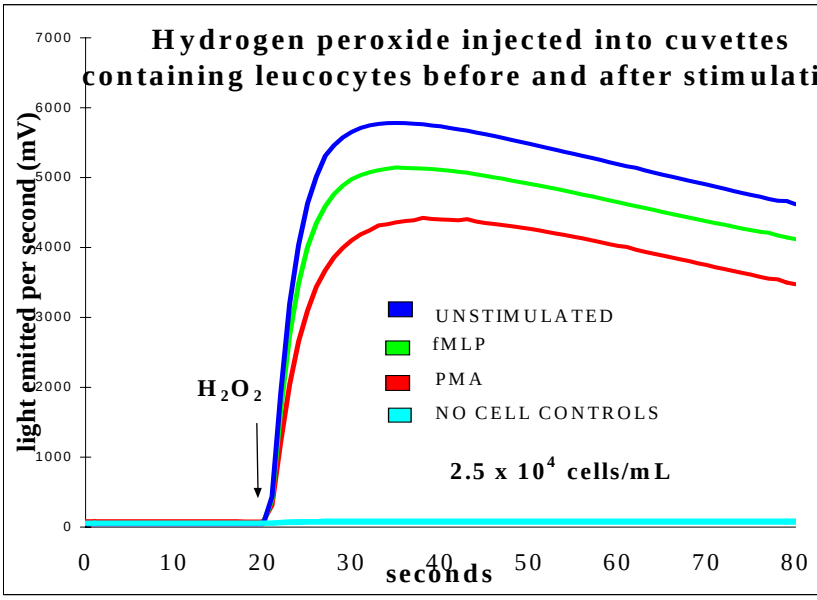


ABEL® Whole Blood Test with Adjuvant-K: following progress of an infection

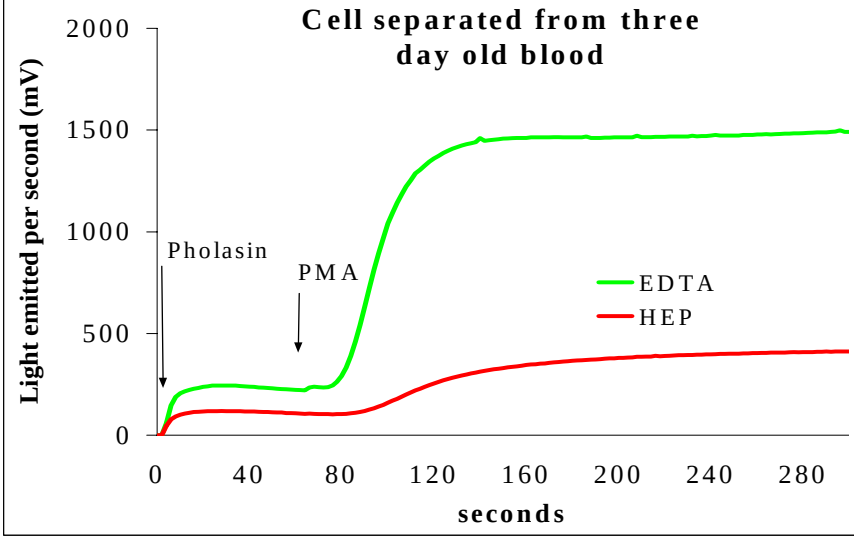




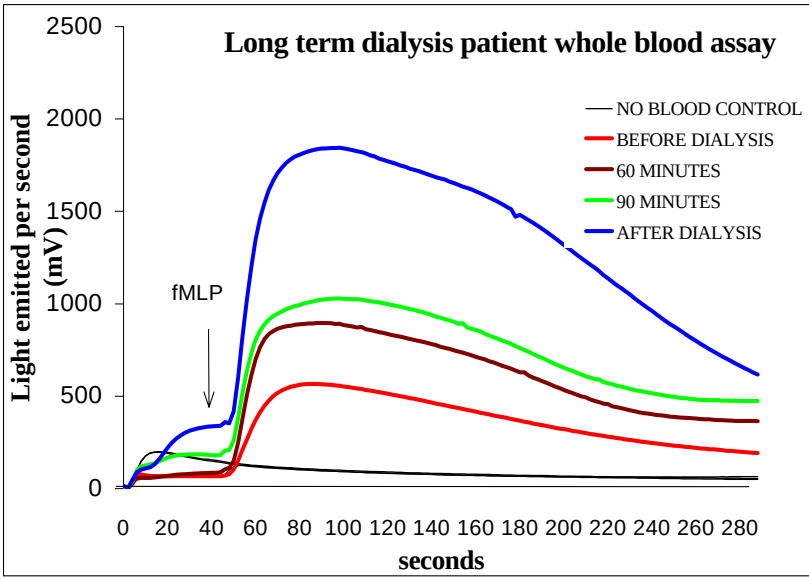
One method for residual granule contents such as myeloperoxidase is to stimulate the cells, either in the diluted whole blood sample or isolated, and inject hydrogen peroxide into the cuvette containing the cells and Phlolasin.



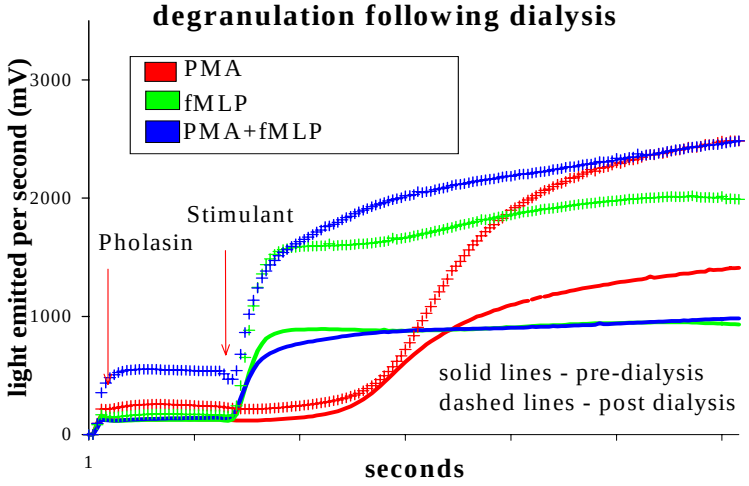
It is possible to identify the degree of degranulation that has occurred



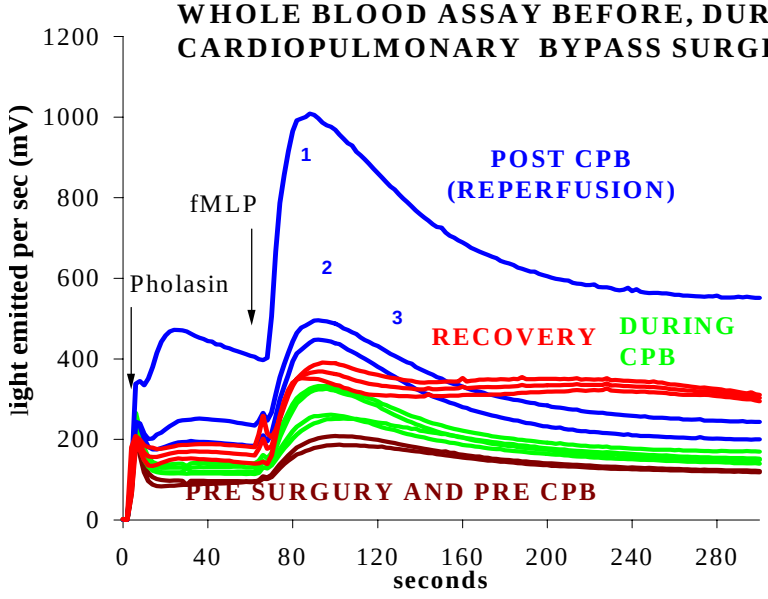
Cells that are still remaining in stored blood will respond to stimuli, even after 3 days. However, cells more readily degranulate when stored in heparin blood and this phenomenon is seen when the cells are separated and the granules washed free from the surface of the cells.



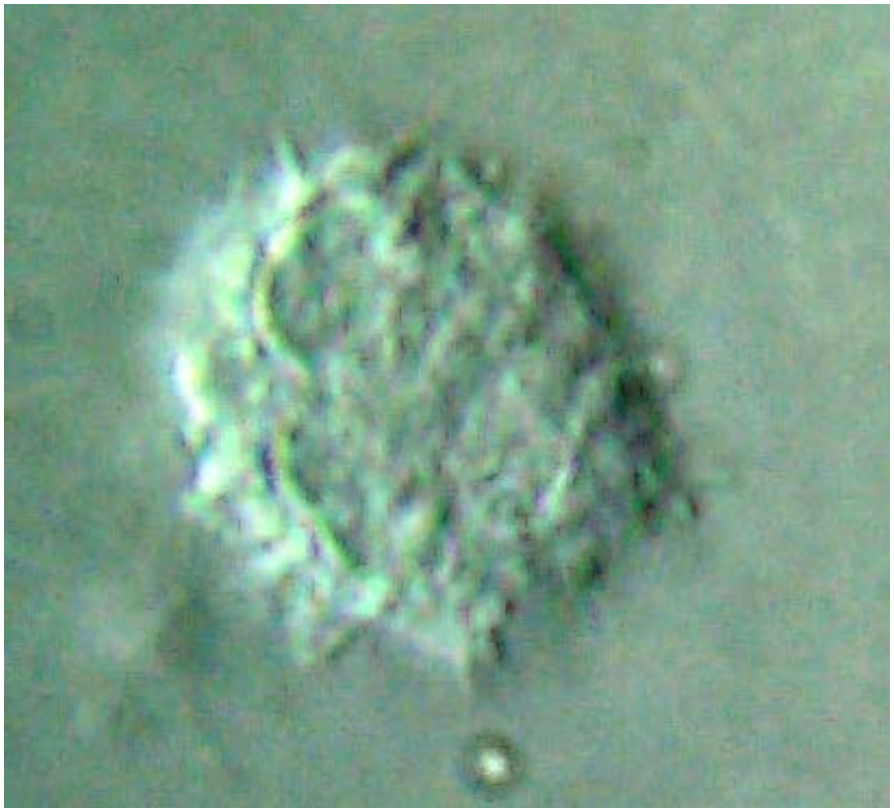
This patient is showing an unusual response to fMLP, even before the start of dialysis. His cells are responding to fMLP in a way approaching the response when cells are stimulated with fMLP and PMA together. This luminescent response is partly due to activation of the NADPH oxidase and partly due to degranulation.
It can be seen in the sample after dialysis that the cells are showing some activation prior to injection of fMLP (blue trace).



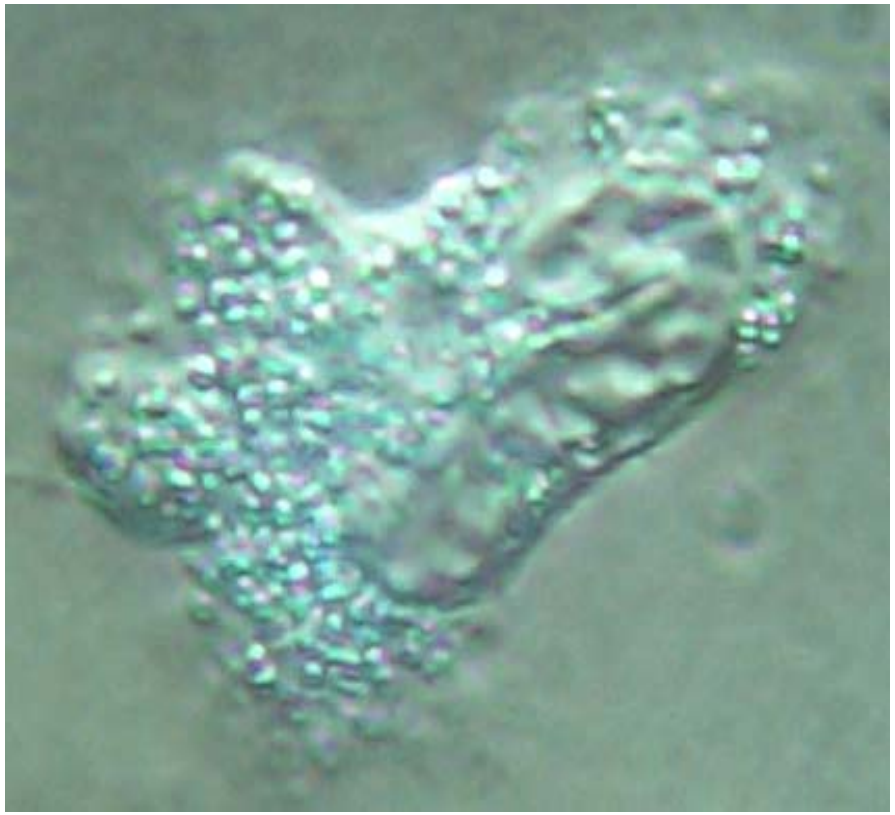
This dialysis patient has cells that are responding to fMLP in the same way his cells respond to a combination of fMLP and PMA. The cells are showing enhanced production of superoxide, evidence of degranulation and at the end of dialysis, activation before stimulation.



This patient had a normal response to fMLP before surgery (dark red curves). During CPB (green curves) no significant change occurred. However, at the time of reperfusion of the heart (blue trace 1) there is an abnormal pre-stimulus signal and an enhanced fMLP response (reminiscent of complement activation). During the recovery 3 days (red) the shape of the curve became abnormal.
This patient suffered serious complications



A. A stimulated neutrophil starting to become active.



B. An advanced stage of activation with the production of free radicals and the release of granules (degranulation)

SOME APPLICATIONS OF THE ABEL® CELL ACTIVATION TEST KITS

ageing
allergies
asthma
atherosclerosis
cardiology
cell biology
clinical engineering
complications of surgery
dermatology
drug efficacy
drug screening
food intolerance
haematology
infection
inflammation
inflammatory bowel disease
multi-organ failure
nephrology
plastic surgery
reperfusion injury
reproductive biology
respiratory distress syndrome (paediatrics)
respiratory medicine
rheumatology
septic shock
smoking
toxicology
tumour killing studies
vascular studies
wound healing

FEATURES AND BENEFITS

FEATURE	BENEFIT
• ability to use whole blood	• no cell separation step minimises time between sample collection and analysis • prevents potential activation of cells during separation • results reflect natural physiological environment of sample
• small sample volume (≡1μL whole blood or a few thousand cells)	• feasible to repeat analysis on same patient • reduces risk to neonates and premature babies • able to carry out long-term experiments on small animals with sequential sampling without sacrificing the animal • ability to carry out a range of experiments on precious cells
• exquisite sensitivity produces detailed kinetic data	• ability to identify unusual pathologies • ability to monitor disease activity • useful for rapid screening of drugs while obtaining detailed kinetic information • ability to monitor response to drug therapy • eliminates the need to measure light accumulated for many minutes and the error associated with such an approach
• identifies primed leucocytes	• useful for disease management • useful in assessing the possible effect a material (or medical device) could have when exposed to different people's blood ❑ select most appropriate medical device ❑ useful in screening new materials • may be valuable in predicting (and perhaps preventing) subsequent adverse reactions ❑ alert medical team ❑ give patient (and family) greater awareness of potential risk ❑ provide defence in the event of litigation
• easy-to-use kits	• require little operator training • reduces technician time
• assay complete within 10 minutes	• ability to analyse many samples within a day

• excellent reproducibility	• confidence in results
• user-friendly format of PMA	• eliminates need for fume cupboards, masks and goggles

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
1. Which anti-coagulant should be used?	Heparin, EDTA or citrate may be used in the tests but cells collected in heparin are more prone to degranulate, especially if they have been primed in some way. This shows up as an enhanced response to PMA in the whole blood test and a reduced response when isolated cells are used.
2. At what temperature should the tests be carried out?	Physiological temperature (37^{o} C for human cells) is recommended although the cells will still respond at lower temperatures.
3. Should I chill the blood after collection?	*a*. For the whole blood assay the sample should be placed inside an insulated container and not subjected to a rapid change of temperature; it should not, for example, be placed on ice. For longer-term storage the blood should be allowed to reach room temperature before being refrigerated. *b*. If work is to be carried out on monocytes that have been isolated from whole blood and then transformed into macrophages, the blood should be chilled immediately to prevent activation of the monocytes.
4. Do I need to use a luminometer that has automatic injectors?	Not necessarily. However, because the reaction of cells to fMLP is rapid, you must inject the fMLP (perhaps with a syringe) while the cuvette is in front of the light detector. Because the lag time for PMA is about 30 seconds the PMA may be injected into the cuvette outside the luminometer and the cuvette then immediately placed into the luminometer.
5. Do I need to mix the sample during the measurement of light?	For the whole blood test the cuvette needs to be mixed frequently and if automatic mixing is not an option then removing the cuvette and mixing with a vortex mixer will give adequate but less than optimal results.
6. Should I use special tubes and cuvettes?	The best material is polypropylene. Glass, unless siliconised, will activate the leucocytes. Polystyrene also sometimes activates the cells.
7. Which cell types respond to the tests?	*a*. Any cells that possess the NADPH oxidase system: neutrophils, eosinopils, monocytes, macrophages, fibroblasts, mesanglial cells and chondrocytes all respond. *b*. Any cells that have xanthine dehydrogenase/oxidase. *c*. Any cells that produce extracellular nitric oxide and superoxide.
8. Blood from which animal species may be used?	Probably all species but we have reports of success with: human, rat, mouse, sheep, cow, horse, pig. It is worthy of note that the response to fMLP may be absent (or very rapid and therefore missed) in some species.
9. What different information do I get	*a*. fMLP does not penetrate the cell but binds to a cell receptor where it initiates a series of events. Cells will show an enhanced

from fMLP and PMA?	response if primed with lipopolysaccharide (LPS) which activates complement. Abnormalities in the fMLP response as measured by Pholasin® (see graphs) are prevalent in long-term dialysis patients, and anecdotal evidence suggests there may be link with allergies, autoimmune diseases, food intolerance and other conditions. *b*. PMA enters the cell. The Pholasin® in the kits provides evidence for the presence of cells with an enhanced propensity to degranulate after stimulation with PMA, by measuring the resultant activity of the NADPH oxidase system and of myeloperoxidase.
10. Can stimulants other than fMLP be used?	Yes. Platelet activating factor, anti-Fc receptor antibodies, LPS, serum, opsonized zymosan, and different sized particles have been tried. Other phorbol esters and other receptor agonists are likely to work in the test.
11. Can I use the Pholasin® in the cell activation kits for measuring antioxidant status?	No. The formulation is different.

RECOMMENDED EQUIPMENT

Luminometer: We recommend a tube or microplate luminometer with automatic mixing/shaking, automatic injection and temperature control.

Syringes and needles: Pholasin®, Adjuvant-P™, fMLP and PMA are supplied sealed under vacuum and are reconstituted to working strength by injecting reconstitution buffer through the rubber insert with a syringe and needle. Do not remove the rubber insert prior to inserting the needle because some powder might escape when the vacuum is released. Needles and syringes are not included in the kits. We recommend using 1 inch, 21 gauge needles and standard 5mL and 10mL syringes.

Blood Collection Tubes: Tubes containing heparin, K_2-EDTA or citrate can be used. As only small volumes of blood are required paediatric tubes can be used.

STORAGE CONDITIONS AND SHELF LIFE

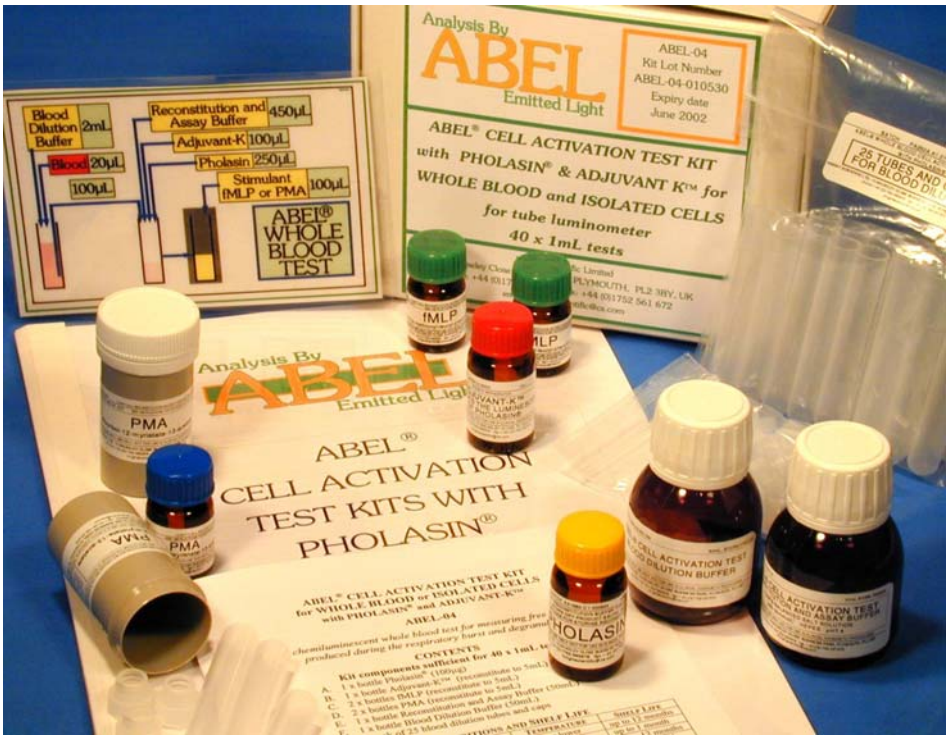
REAGENT	FORMAT	TEMPERATURE	SHELF LIFE
PHOLASIN®	freeze dried	-20°C or lower	up to 12 months
	reconstituted	-20°C or lower	up to 1 month
fMLP	freeze dried	-20°C or lower	up to 12 months
	reconstituted	-20°C or lower	up to 1 month
PMA	freeze-dried reconstituted	-20°C or lower 2-8°C	up to 12 months up to 3 days
ADJUVANT-K™	freeze dried	-20°C or lower	up to 12 months
	reconstituted	2-8°C	up to 3 month
BLOOD DILUTION BUFFER	liquid	-20°C or lower	up to 12 months
	liquid	2-8°C	up to 1 month
RECONSTITUTION & ASSAY BUFFER	liquid	-20°C or lower	up to 12 months
	liquid	2-8°C	up to 1 month

ORDERING INFORMATION

	Catalogue Number
Whole Blood Cell Activation Kits with Adjuvant-K™	KSL-ABEL-04 (40 x 1mL tests)
	KSL-ABEL-05 (80 x 1mL tests)
	KSL-ABEL-06 (120 x 1mL tests)
M = microplate kits	KSL-ABEL-04M (200 x 200µL tests)
	KSL-ABEL-04M2 (2 bottles Pholasin)

Please enquire about ABEL® Whole Blood Kits with Adjuvant-P ™

	Catalogue Number
Isolated Cell Activation Kits with Adjuvant-K™	KSL-ABEL-14 (40 1mL tests)
	KSL-ABEL-15 (80 x 1mLtests)
	KSL-ABEL-16 (120 x 1mLtests)
M = microplate kits	KSL-ABEL-14M (200 x 200µL tests)
	KSL-ABEL-14M2 (2 bottles Pholasin)



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