For further help and advice, please contact:

Knight Scientific Limited
15 Wolseley Close Business Park,
PLYMOUTH, PL2 3BY, UK
telephone: +44 (0) 1752 565 676
fax : +44 (0) 1752 561 672

e-mail: Info@knightscientific.com
www.knightscientific.com

OXIDATIVE STRESS KIT

ABEL® CELL ACTIVATION TEST KIT for WHOLE BLOOD or ISOLATED CELLS with PHOLASIN® and ADJUVANT-K™

Microplate Luminometer Kit ABEL-00M

A chemiluminescent whole blood test for measuring free radicals produced during the respiratory burst and degranulation

CONTENTS

Kit components sufficient for 100 x 200µL tests

A. 1 x bottle Pholasin® (50µg)
B. 1 x bottle Adjuvant-K™ (reconstitute to 5mL)
C. 1 x bottle fMLP (reconstitute to 2.5 mL)
D. 2 x bottles PMA (reconstitute to 2.5 mL)
E. 1 x bottle Reconstitution and Assay Buffer (25mL)
F. 2 x bottles Blood Dilution Buffer (50mL)
G. 2 packs of 25 blood dilution tubes and caps
H. 1 x 96 well white luminometer microplates

STORAGE CONDITIONS AND SHELF LIFE

REAGENT	FORMAT	TEMPERATURE	SHELF LIFE
PHOLASIN® (Cell Activation)	Freeze dried	-20°C or lower	up to 12 months
	Reconstituted	-20°C or lower	up to 1 month
RECONSTITUTION & ASSAY BUFFER FOR PHOLASIN®	Liquid	-20°C or lower	up to 12 months
		2-8°C	up to 1 month
BLOOD DILUTION BUFFER	Liquid	-20°C or lower	up to 12 months
		2-8°C	up to 1 month
ADJUVANT-K™ (Reconstitute to 5mL)	Freeze dried	2-8°C	up to 12 months
	Reconstituted	2-8°C	up to 2 months
fMLP (Reconstitute to 2.5mL)	Freeze dried	-20°C or lower	up to 12 months
	Reconstituted	-20°C or lower	up to 1 month
PMA (Reconstitute to 2.5mL)	Freeze dried	-20°C or lower	up to 12 months
	Reconstituted DO NOT FREEZE	2-8°C DO NOT FREEZE	2 days DO NOT FREEZE

If any packs are damaged or bottles appear to have leaked, do not use the items, but contact your supplier for advice.

This kit is supplied for research use only. Responsibility will not be accepted for misuse of the kit components. This kit contains glass items, and the use of sharps is recommended; these should be handled with due care and disposed of correctly according to good laboratory practice.

This kit has been developed for use with diluted whole blood to measure extracellular free radicals produced by leucocytes during the respiratory burst. It can also be used to measure free radicals produced by isolated leucocytes and other types of cell.

The kit contains sufficient reagents for 100 tests of 200µL each and sufficient tubes for making 50 dilutions of whole blood.

A microplate luminometer with shaking, temperature control (37°C) and automatic injectors is ideal. However, if your luminometer does not shake then it is suggested that the microplate should be agitated externally immediately before placing in the luminometer. Manual injection can be used with PMA if careful attention is given to timing, however with fMLP when the lag time from stimulus to response is 2-5 seconds is difficult

Pholasin®, Adjuvant-K™, fMLP and PMA are all supplied in vials that have been sealed under vacuum. **IT IS IMPORTANT THAT YOU DO NOT REMOVE THE RUBBER INSERT UNTIL THE BOTTLES HAVE BEEN RECONSTITUTED WITH BUFFER THAT HAS BEEN INJECTED THROUGH THE SEPTUM.**

PROTOCOL

Reconstitution of Pholasin®

The Pholasin® has been specially formulated to be reconstituted to 5mL giving a final concentration of 10µg mL^{-1} in a precise formulation of buffer. NOTE: reconstituting the Pholasin® with a different volume will give variable results.

It is important to follow these instructions to ensure that the contents of the vial are not lost when the vacuum in the bottles is released.

1. Take up 5 mL Reconstitution and Assay Buffer into a syringe (not supplied).
2. Fit a needle (1inch, 21 gauge; 0.8 x 25mm) to the syringe.
3. Remove the protective screw cap from the vial of Pholasin® (A). Carefully push the needle through the rubber septum. When the septum is pierced, the syringe contents will be drawn into the vial.
4. Discard the needle (using good laboratory practice).
5. Invert and roll the bottle at least 5 times to dissolve the contents.
6. The Pholasin® has now been reconstituted to 10µg mL^{-1}. For each 200µL test 50µL is required. Remove the required amount for the number of tests to be undertaken to a clean bottle or tube.
7. Freeze the remaining reconstituted Pholasin® at <-20°C. This should be used within 1 month. Loss of activity may occur every time the Pholasin® is thawed, therefore, you might wish to divide the reconstituted Pholasin® into a number of samples prior to freezing.

Reconstitution of Adjuvant-K™

Adjuvant-K™ enhances the luminescence of Pholasin® during assays with diluted whole blood. Reconstitution with 5 mL of buffer will give you enough for 250 tests; 20µL is used in each 200µL test.

1. Take up 5 mL Reconstitution and Assay Buffer into a syringe.
2. Fit a needle to the syringe.
3. Remove the protective screw cap from the vial of Adjuvant-K™ (B). Carefully push the needle through the rubber septum. When the septum is pierced, the syringe contents will be drawn into the vial.

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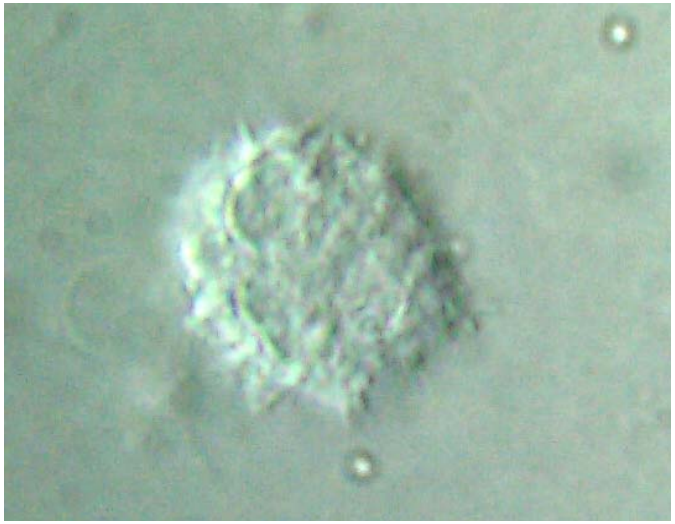
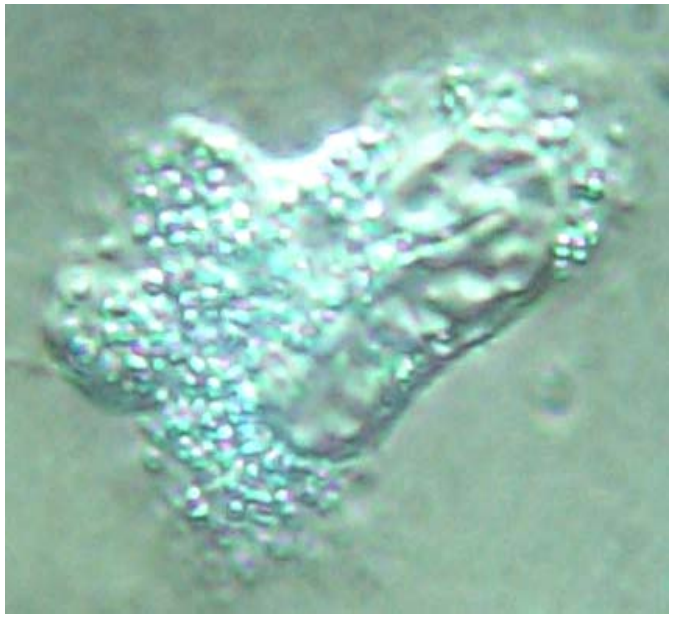
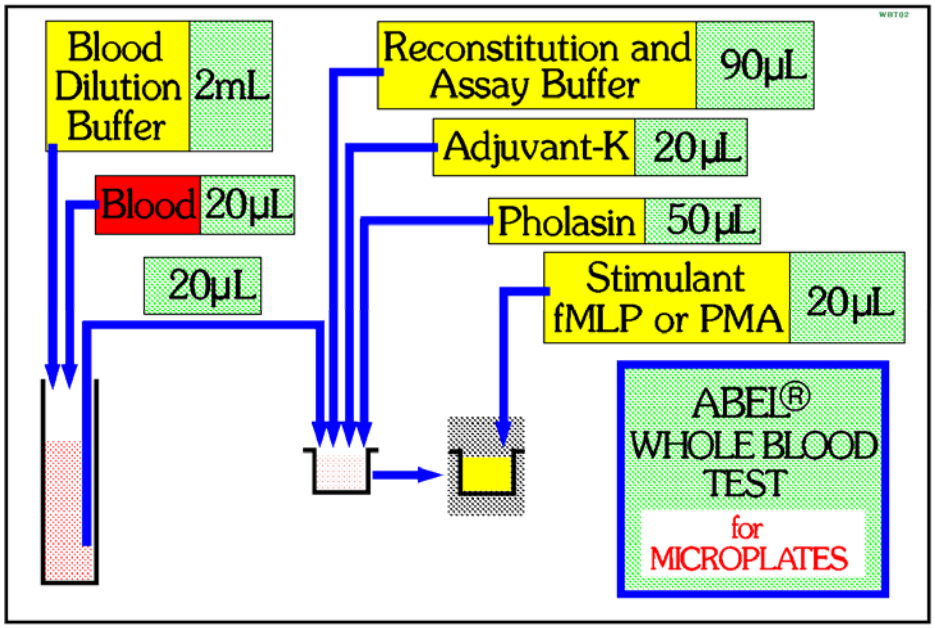
A)



B)



(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).



SUMMARY

To an opaque white microplate well add:

a) 90µL RECONSTITUTION AND ASSAY BUFFER
b) 20µL RECONSTITUTED ADJUVANT-K™
c) 50µL PHOLASIN®
d) 20µL DILUTED WHOLE BLOOD

1. For a control use 20µL plasma diluted 1:100 with blood dilution buffer.
2. Mix contents of well or shake plate.
3. Place the plate (or strip) in luminometer; incubate at 37°C, (with shaking if possible) for 1 minute.
4. Inject 20µL (or 25µL if this is minimum capable; reduce buffer to 85µL accordingly) stimulant (PMA or fMLP).
5. Continue measuring light emitted.

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4. Discard the needle (by approved laboratory practice) but save the syringe; it can be used to reconstitute the other vials.
5. Invert and roll the bottle at least 5 times to dissolve the contents.
6. The Adjuvant-K™ has been reconstituted to 1 enhancing unit per 100µL. For each 200µL test 20µL is required. Remove the required amount for the number of tests to be undertaken to a clean bottle or tube.
7. Store the remaining reconstituted Adjuvant-K™ at 2-8°C. This should be used within 2 months.

Reconstitution of fMLP

AS THE CHEMICAL, PHYSICAL AND TOXICOLOGICAL PROPERTIES OF fMLP HAVE NOT BEEN THOROUGHLY INVESTIGATED PLEASE EXERCISE DUE CARE.

NOTE: If the lowest injection volume of the luminometer is 25µL, then reconstitute the vial of fMLP with 3.1mL of R&A Buffer to obtain a concentration of 10µmol L^{-1}. If the luminometer can inject 20µL reconstitute the vial with 2.5mL as instructed below:

1. Take up 2.5 mL Reconstitution and Assay Buffer (as before) into a syringe.
2. Fit a new needle to the syringe.
3. Remove the protective screw cap from the vial of fMLP (C). Carefully push the needle through the rubber septum. When the septum is pierced, the syringe contents will be drawn into the vial.
4. Discard the needle (by approved laboratory practice) but save the syringe; it can be used to reconstitute the other vials.
5. Invert and roll the bottle at least 5 times to dissolve the contents.
6. The fMLP has been formulated in such a way that on reconstitution the bottle contents will be at a concentration of 10µmol L^{-1}. For each 200µL test 20µL is required giving a final concentration of 1µmol L^{-1}. Remove the required amount for the number of tests to be under taken to a clean bottle or tube.
7. Freeze the remaining reconstituted fMLP at <-20°C. This should be used within 1 month. Loss of activity is likely to occur every time the fMLP is thawed, therefore you might wish to divide the reconstituted fMLP into a number of samples prior to freezing.

Reconstitution of PMA: DO NOT FREEZE RECONSTITUTED PMA

THE RECONSTITUTED PMA IS AT A VERY LOW CONCENTRATION. PMA, HOWEVER, IS HIGHLY TOXIC BY INHALATION, CONTACT WITH SKIN AND IF SWALLOWED. IT IS IRRITATING TO EYES, RESPIRATORY SYSTEM AND SKIN. IT IS A POSSIBLE CARCINOGEN. IN CASE OF AN ACCIDENT OR IF YOU FEEL UNWELL, SEEK MEDICAL ADVICE. IN CASE OF CONTACT WITH EYES, RINSE IMMEDIATELY WITH PLENTY OF WATER. PROTECTIVE GLOVES AND EYE PROTECTION IS RECOMMENDED.

PMA (Phorbol-12-myristate-13-acetate), when reconstituted with 2.5mL Reconstitution and Assay Buffer, will give a concentration of 8µmol L^{-1} (5µg mL^{-1}). For each 200µL test 20µL is used to give a final concentration of 0.8µmol L^{-1} (0.5µg mL^{-1}). PMA CANNOT BE FROZEN ONCE IT HAS BEEN RECONSTITUTED. IF STORED IN THE REFRIGERATOR IT CAN BE USED FOR ABOUT 2-3 DAYS. [EXTRA BOTTLES OF PMA CAN BE ORDERED].

NOTE: If the lowest injection volume of the luminometer is 25µL, then reconstitute the vial of PMA with 3.1mL of R&A Buffer to obtain a concentration of 8µmol L^{-1}. If the luminometer can inject 20µL reconstitute with 2.5mL as instructed below:

1. Take up 2.5 mL Reconstitution and Assay Buffer into a syringe.
2. Fit a needle to the syringe.
3. Remove the protective screw cap from the vial of PMA (D). Carefully push the needle through the rubber septum. When the septum is pierced, the syringe contents will be drawn into the vial.
4. Discard the needle (by approved laboratory practice) but save the syringe; it can be used to reconstitute the other vials.
5. Invert and roll the bottle at least 5 times to dissolve the contents.

fMLP and PMA mixed

If fMLP and PMA are to be mixed before use in the assay, then the PMA can be reconstituted with 2.5mL (or 3.1mL) reconstituted fMLP (10µmol L^{-1}). Alternatively 20µL (25µL) reconstituted fMLP and 20µL (25µL) PMA can be added at the same time with 20µL (25µL) less assay buffer added to the luminometer cuvette before the start of the assay (see test procedure below).

Collection and Dilution of Blood

1. Collect sterile blood samples in tubes containing EDTA or heparin.
2. Blood should preferably be assayed on the day of collection but results can be obtained up to 2 days after collection if the blood is chilled to 4°C.
3. Add 2mL of Blood Dilution Buffer to one of the empty tubes. Add 20µL of heparin- or EDTA- blood. Cap the tube and gently invert 3 times to mix. Diluted blood should be used within 15 minutes. If more assays are required on the same blood then a fresh dilution should be made.
4. For each 200µL test use 20µL diluted whole blood to obtain a 1:1000 final dilution.

TEST PROCEDURE FOR DILUTED WHOLE BLOOD

If the luminometer has three automatic injectors then the reconstituted Pholasin® should be put in one injector, the fMLP in another and the PMA in the third. **NOTE THAT IF PMA IS USED IN AN INJECTOR IT IS IMPORTANT THAT THE INJECTOR IS RINSED AFTER USE AT LEAST 5 TIMES EACH WITH WATER, DETERGENT, WATER, 50% ISOPROPYL ALCOHOL OR ETHYL ALCOHOL FOLLOWED BY WATER.**

The assay can be performed manually, but it is important to inject the fMLP very quickly as the respiratory burst (activation of the NADPH oxidase system) usually commences within 2-5 seconds.

1. To an opaque white microplate well add:
   - 90µL RECONSTITUTION AND ASSAY BUFFER (or 85µL if inject 25µL stimulant)
   - 20µL RECONSTITUTED ADJUVANT-K™
   - 50µL PHOLASIN®
   - 20µL DILUTED WHOLE BLOOD
2. For a control use 20µL plasma diluted 1:100 with blood dilution buffer.
3. Mix contents of well or shake plate.

Continuous Measurement

1. Place the plate (or strip) in the luminometer which is at 37°C, and incubate (with shaking if possible) for 1 minute. No more than 5 wells should be filled at any one time.
2. If the luminometer does not have a shaker then immediately after the 1 minute incubation step, remove the plate and mix/shake externally.
3. As an alternative you can inject 50µL reconstituted Pholasin® into the microplate well in the pre-measure position (glow) after the incubation step.
4. Record the light emitted from the sample continually for 1 minute.
5. After 1 minute inject 20µL (25µL) of either fMLP, PMA, or a mixture of the two stimulants, from an automatic injector.
6. Record the emission of light continuously for a further 4 minutes.

Repeat Measurements Using a Number of Wells

1. Set up the assay as described above.
2. Determine the interval time between wells and determine the maximum number of wells that can be measured in which the interval time is less than 5 seconds when fMLP is used as stimulant and less than 20 seconds when PMA is used.

NOTE: THE INTENSITY OF THE LIGHT EMITTED WILL BE REDUCED IN THE ABSENCE OF MIXING; DO NOT COMPARE THE RESULTS OF ASSAYS CARRIED OUT WITH AND WITHOUT MIXING.

TEST PROCEDURE FOR ISOLATED CELLS

1. The test procedure is identical to the whole blood assay with 20µL cell suspension replacing 20µL 1:100 diluted blood.
2. Assays with isolated leucocytes are usually performed with the mixer off.

NOTE: PIG NEUTROPHILS DO NOT APPEAR TO HAVE RECEPTORS FOR fMLP OR THE RESPONSE MAY BE VERY RAPID AND WAS REPORTED AS MISSING. IT HAS BEEN SUGGESTED THAT OTHER ANIMAL SPECIES MAY ALSO LACK SUCH RECEPTORS OR HAVE VERY SHORTER LAG TIMES. PLATELET ACTIVATING FACTOR COULD BE SUBSTITUTED FOR fMLP IN SUCH ASSAYS.