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BACTERIAL CONTAMINATION SCREENING KIT

Microbiotest for ultra-rapid on-site screening of water for contamination by bacteria and biological residues

STANDARD OPERATIONAL PROCEDURE

BACTERIAL CONTAMINATION SCREENING MICROBIOTEST

RESULTS SHEET

Type of water sample : ……………………………………………………………….

Sampling site : ……………………………………………………………………..

Date : ………………………………………………………………………………….

Name of operator : ………………………………………………………………

Type of test : □ Direct test □ Membrane filter test

Direct test RLU score : ………..

RLU	Degree of contamination
○ < 50	○ Very low
○ < 200	○ Low
○ 200 - 1000	○ Significant
○ > 1000	○ Very high

Membrane filter test

Volume of water filtered : ……….. ml

RLU score : ………..

Number of RLU per ml = Total RLU score / ml water filtered = …….. RLU

	Degree of bacterial contamination	
○ < 200	○ Very low	-
○ < 1000	○ Relatively low	+
○ > 1000	○ Significant	++
○ > 5000	○ High	+++
○ > 10.000	○ Very high	++++

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INTRODUCTION TO THE BACTERIAL CONTAMINATION SCREENING MICROBIOTEST

ORIGIN

The development of the Bacterial Contamination Screening Kit has been instigated by the worldwide demand for "field tests" for very rapid detection of bacteria in water.
The research performed in the company MicroBioTests Inc. eventually led to the elaboration of this practical, user-friendly and low cost "field microbiotest". This test can be performed "anytime and anywhere" with the aid of a small low cost portable luminometer incorporated in a small lightweight case which contains specific materials for performing the assay.

The equipment and materials included in the case also allow to perform in parallel "Toxi-Screening" microbiotests to evaluate the toxic hazard of suspect water samples.

BIOLOGICAL BACKGROUND OF THE ASSAY

Natural waters all contain living organisms which - like all living creatures on earth - will at one moment of time die and be decomposed by bacteria.

The amount of microbes in water is therefore a signal for the quantitative importance of bacterial decomposition of biological residues.

The energy source of all living organisms, including bacteria, is ATP (Adenose TriPhosphate), and the Bacterial Contamination Screening test measures the total amount of ATP in a water sample, as a quantitative criterion for the presence of bacteria and biological residues.

The total ATP content of a water sample comprises the "intracellular" ATP from all living biota and biological residues present in the sample, and the (dissolved) "extracellular" ATP released from dead organisms.

ATP in water can be measured by chemical luminescence through a luciferin/luciferase reaction with a specific chemical reagent.

9. Repeat steps 7 and 8 a few times to eliminate all traces of decontaminating chemical from the syringe and the filter holder.

10. Disconnect again the syringe from the filter holder, and fill it with air.

11. After reconnecting the syringe with the filter holder, flush the air through the filter holder to eliminate all water residues.

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The following very simple and rapid decontamination procedure can be applied in the field with the materials contained in the"Decontamination Box".

Procedure

1. Fill the 2 cups from the Decontamination Box with mineral water fom a freshly opened bottle.

2. Open the vial with the Decontamination Chemical, take one of the small tablets with the plastic pincette and drop it into the cup labelled "Decontamination Solution"

N.B. The chemical selected for the rapid decontamination of the syringe and the filter holder is "sodium dichloroisocyanurate" (NaDCC), a commonly used low risk disinfection compound which releases chlorine when dissolved in water.

3. Wait a few minutes untill the chemical is dissolved completely.

4. Disconnect the filter holder from the syringe, put the tip of the syringe in the decontaminating solution and fill the syringe.

5. Connect the syringe again with the filter holder and after about 1 minute, slowly flush the decontamination solution through the filter holder.

N.B. In case several prefilter tests are carried out one after the other, the prefilter (with the non-bacterial material on the outside) can be left in the filter holder during the decontamination operation.
Flushing of the decontamination solution through the prefilter will indeed also automatically clean the prefilter.

6. Repeat steps 4 and 5 a few times to eliminate all the bacteria from the syringe and the filter holder.

7. Disconnect again the filter holder from the syringe, and fill the latter with water from the Clean Water vial.

8. Reconnect the syringe with the filter holder and slowly flush the clean water through the filter holder.

The amount of light produced is proportional to the total ATP content of the water sample, and is measured in a luminometer and expressed in RLU (Relative Light Units).

PRINCIPLE

The Bacterial Contamination Screening Kit contains tubes with ATP reagents which, in combination with the equipment and materials contained in a "Luminescence Measurements Case", allow to perform two distinct types of analyses :
- a direct test which measures the "total amount of ATP" in the analysed water sample
- a "membrane filter" test which only measures the ATP from the living bacteria present in the water sample, after prior elimination of all intra- and extracellular ATP from other biota and biological residues.

SCOPE

The Bacterial Contamination Screening microbiotest is suited for a variety of analyses such as : microbial contamination of surface and groundwaters, routine microbial screening of drinking waters, swimming pools and aquaculture tanks, water contamination emergencies, and in situ follow up of the efficiency of various kinds of bacteriological water treatments

ASSETS OF THE BACTERIAL CONTAMINATION SCREENING MICROBIOTEST

1. The "ready to use" tubes with the ATP reagents make this field test ultra simple and rapid in comparison to any other ATP measurement method.
2. Contrary to conventional microbiological techniques which are all "laboratory based" and which are dependent on special equipment and materials and require one to several days incubation, "Bacterial Contamination Screening microbiotests" can be carried out anytime and anywhere in the field in a few minutes of time. The assays only require transportation of the kit and the small lightweight Luminescence Measurements Case to the site of investigation.
3. The bacterial contamination screening tests can be performed at ambient temperature with maximum luminescence scorings in the

range 15 °C to 25 °C.
4. The membrane filter test procedure option specifically reflects the degree of contamination of the suspect water by bacteria, after prior elimination of the ATP from other biota and biological residues.
5. The Bacterial Contamination Screening microbiotest can also be applied to estuarine and marine waters to detect their degree of contamination by freshwater and/or marine bacteria.

FEATURES

Each Bacterial Contamination Screening Kit contains 10 “Unit Boxes” with 2 tubes with ATP reagents and two 200 µl finntips in each box. Each tube allows for the measurement of one water sample.

The tubes with ATP reagents are composed of 2 distinct compartments separated by a partition (*see Figure 1*) :
- an upper chamber containing a liquid ATP extractant
- a bottom chamber containing a solid ATP reagent

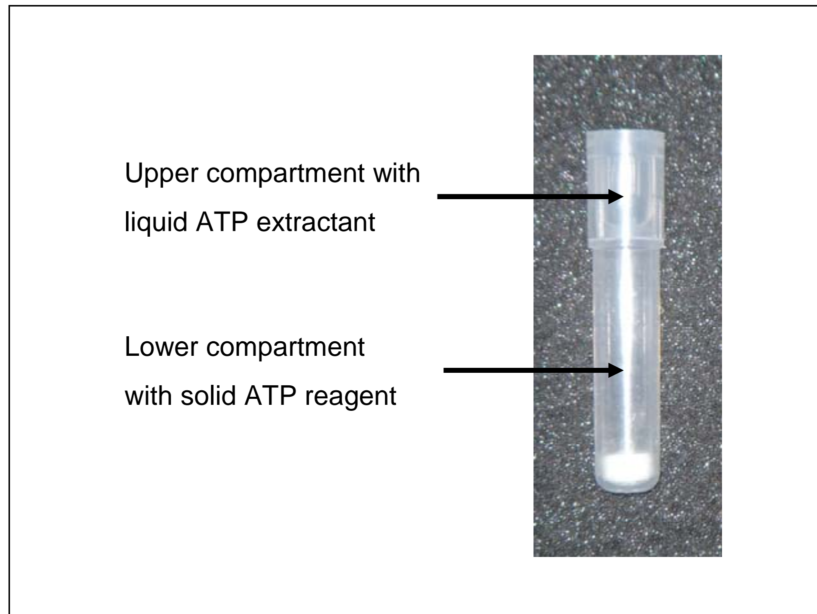

Figure 1 : Tube with ATP reagents

The testing procedure is similar to that detailed above for the membrane filter method, and shall be applied by first following steps 1 to 10.

After flushing the seawater through the 2 connected filter holders, the membrane filter is taken out from its holder with the pincette, and dipped 2-3 times, but very rapidly, in (clean) freshwater (e.g. mineral water or tapwater) to eliminate the remaining salts from the membrane filter.

The experimental procedure is then continued from steps 11 to 16 of the membrane filter method detailed above.

Verification step
The degree of microbial contamination of a water detected in a few minutes by the former two screening methods, can subsequently be verified by a simple “plate count ” procedure on small discs containing a bacterial nutrient agar.
The inoculation of a “Verification disc for total bacterial count” is carried out in the field, concurrently with the direct or membrane filter screening assays, by simply hydrating the nutrient agar in the disc with 1 ml of the water sample. Subsequently the disc is incubated in the laboratory either at room temperature (several days) or at 35°C (24h).
The growth of the bacteria eventually leads to tiny red colonies which are clearly visible on the disc. The number of microbial colonies (Colony Forming Units = CFU) is representative for the degree of bacterial contamination of the water.
N.B. Other discs with special growth media can also be used to detect specific pathogenic bacteria (E.coli, coliforms, Salmonella, etc.).

DECONTAMINATION OF THE SYRINGE AND THE FILTER HOLDERS

After application of the membrane filter test procedure, the syringe and the 2 filter holders need to be “decontaminated” from the bacteria which may remain adsorbed on the inside walls.

In order to avoid interferences from these “leftovers”, both the syringe and the membrane filter holder have to be “decontaminated” after each membrane filter test.

At the end of the test, take out the prefilter from the second filter holder and discard it.

<u>Evaluation of the degree of microbial contamination of water samples</u>

Since the volumes of water flushed through the membrane filter will mostly differ from one sample to the other, <u>the RLU scores are first recalculated to 1 ml water sample</u> by dividing the scored RLU figure by the number of ml water flushed through the filter holders.

A gross ranking of the amount of bacteria present in 1 ml of the analysed water can be made as follows :

< 200 RLU	: only a few bacteria	-
< 1000 RLU	: relatively low number of bacteria	+
> 1000 RLU	: significant number of bacteria	++
> 5000 RLU	: high bacterial contamination	+++
> 10.000 RLU	: very high bacterial contamination	++++

N.B. The former figures are indicative and should not be interpreted strictly quantitatively, but preferably by – and + signs as shown above, to express the importance of the microbial contamination.

<u>Determination of the degree of microbial contamination of estuarine and marine waters</u>

The Bacterial Contamination Screening Kit can also be used for the evaluation of the degree of seawater contamination by marine as well as by freshwater bacteria.

Salinity, however, interferes with the ATP reactions, <u>so only the membrane filter method can be applied, in combination with an extra step to eliminate the salts remaining on the filter after flushing the water sample through the filter holders.</u>

When applied to estuarine or marine water samples, the membrane filter procedure - like its freshwater counterpart - also only measures the "intracellular" microbial ATP from the contaminating bacteria after elimination of all other "interfering" ATP.

The "Luminescence Measurements Case" contains the portable luminometer and 2 holders for the tubes with ATP reagents, as well as various other items for performance of membrane filter tests. The Luminescence Measurements Case furthermore also contains materials to perform Toxi-Screening tests.

SHELF LIFE

<u>The Unit Boxes with the tubes with the ATP reagents must be stored in the refrigerator at temperatures between 2°C and 8°C, but should not be frozen !</u>

If stored properly the shelf life of the tubes with the ATP reagents is 6 months to 1 year.

SENSITIVITY AND DETECTION THRESHOLD

The ATP luminescence measurement is very sensitive - each ATP molecule indeed produces one photon of light - and the portable luminometer hence signals the presence of quite low bacterial numbers (less than 100 bacteria per ml).

N.B. It may be noted that 100 bacteria per ml (100.000 per liter) is the norm used in many countries for the maximum number of "not pathogenic" microbes allowed in drinking water.

SPECIFICITY

ATP measurements are "not specific" for any particular kind of bacteria and hence **<u>do not give any indication on the presence or the absence of pathogenic bacteria</u>** (coliforms, salmonella's etc…) !

The intensity of the measured luminescence in turn gives an ultra-rapid signal for the overall degree of contamination of the analysed water by microbes and biological residues, which allows to take immediate decisions on the suitability of the concerned water for specific uses, and/or the need for treatment.

N.B. "Plate count tests" on small discs with specific bacterial growth media can be started "in situ" immediately after the bacterial contamination screening assays. These tests allow to verify in 24 hours the importance of the bacterial contamination of the analysed waters as revealed by the rapid on-site tests. Additional plate count tests on special discs can also be performed to detect and quantify the presence of pathogenic bacteria such as E.coli, coliforms, Salmonella, etc..

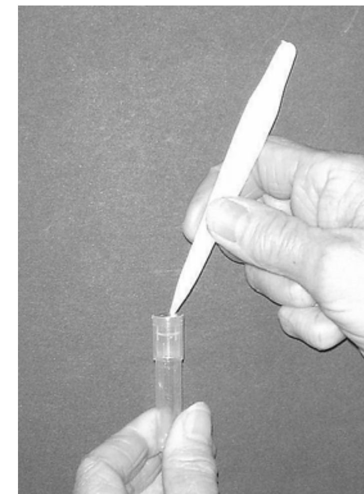

Figure 14

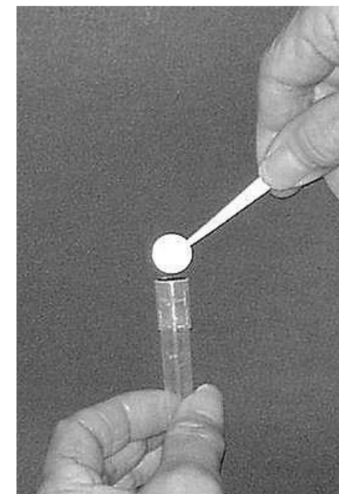

Figure 15

N.B. The flushing of the water sample through the connected filter holders has 3 distinct effects :

a) it eliminates all the (dissolved) extracellular ATP and interfering dissolved chemicals present in the analysed water sample.

b) it captures all the solid residues and all the (non bacterial) biota on the (10 µm) prefilter

c) it captures all the bacteria (which are easily passing through the prefilter) on the (0.2 µm) membrane filter.

11. With the aid of the pincette, tear the top cover of the tube with ATP reagents open and remove this cover completely (*Figure 14*).

12. Disconnect the two filter holders, open the holder with the membrane filter and take out the membrane filter with the aid of the pincette.

N.B. The membrane filter can easily be removed by inserting one of the tips of the pincette in the Luer opening of the hollow part of the membrane filter holder and pushing the membrane filter upwards.

13. Insert the membrane holder in the upper compartment of the tube containing the ATP extractant (*Figure 15*). Make sure that the entire surface of the membrane filter is in good contact with the liquid ATP extractant and move the filter around with the pincette to ensure a maximum contact of the ATP extractant with the bacteria trapped on the membrane filter.

N.B. Never use a metal pincette to perform these manipulations since the chemical reaction of the ATP extractant with the metal will seriously affect the subsequent RLU readings !

14. After about 2 minutes, put a 200 µl finntip on the 200 µl Finnpipette and insert the tip into the compartment with the ATP extractant, along the membrane filter

15. Suck up 200 µl of the liquid and slowly push the tip of the finntip further down in the tube, through the partition separating the two compartments. Take care during this operation not to spill liquid through the opening at the top of the tube.

16. Proceed further as indicated in steps 5 to 11 of the “Direct test” outlined above.

CONTENTS OF THE BACTERIAL CONTAMINATION SCREENING KIT

Unit Boxes
10 boxes with 2 tubes with ATP reagents and 2 finntips (200 µl).

Results sheets
20 sheets for the scoring of the luminescence data of the direct test or the membrane filter test, and the evaluation of the degree of bacterial contamination of the analysed water.

Standard Operational Procedure manual
A detailed brochure with all the instructions and illustrations for the performance of the direct test and the membrane filter test.

Bench protocol
An abbreviated version of the Standard Operational Procedure manual.

Specification sheet
A sheet indicating the batch number and the shelf life of the tubes with ATP reagents.

CONTENTS OF THE LUMINESCENCE MEASUREMENT CASE

The Luminescence Measurements Case contains the portable luminometer and specific materials for performance of the two test procedures. The case also contains various other items for performance of “Toxi-Screening” microbiotests.

1. Portable luminometer
Small and lightweight luminometer (20 x 8 x 5 cm; 0.3 kg) with a digital display for direct reading of the luminescence in “Relative Light Units” (RLU).

Figure 2 : Contents of the Luminescence Measurements Case

The luminometer is a Lumitester PD-10 instrument from Kikkoman Corporation, and is provided with 2 AA (or R6) alkaline batteries, for use in the field. The luminometer can be connected to the electrical mains with an AC connector and to a printer or a computer with a specific connector.

2. Holes for the tubes with ATP reagents
The holes in the foam insert layer of the Luminescence Measurements Case allow to keep the tubes with the ATP reagents in a vertical position during the manipulations.

3. Syringe
A 10 ml synthetic syringe with Luer fitting, for filtering selected volumes of water through the prefilter and the membrane filter in the connected filter holders, for performance of the membrane filter test procedure.

Figure 12

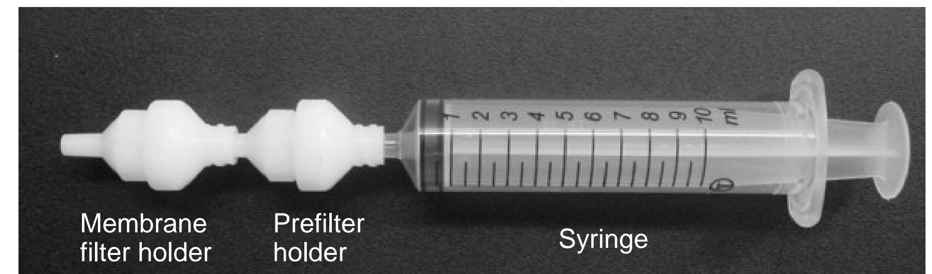

Figure 13

3. With the pincette take a 0.2 µm membrane filter from the box with membrane filters, put it in the hollow compartment of the second filter holder and reassemble tightly the 2 parts of the filter holder.

4. Connect the filter holder containing the prefilter to the filter holder which contains the membrane filter, by inserting the tip of the former into the Luer-opening of the latter (*Figure 11*).

N.B. Don't touch the membrane filter with the fingers during these operations to avoid contamination of this filter by ATP (always) present on our skin !

5. Take the 10 ml syringe and fill it with a (preselected) volume of water sample (*Figure 12*).

N.B. The volume of water to be used is dependent of 2 factors :
a) the amount of "suspended solids" in the water, which may clog the prefilter (even after filtration of only a few ml water sample)
b) the degree of microbial contamination of the water which, in case of very low bacterial numbers, will necessitate to flush more than 10 ml water sample through the membrane filter, to obtain "a meaningful" RLU luminescence figure.

Determination of the most appropriate volume of water sample may therefore necessitate a few consecutive trials and measurements.

6. Insert the tip of the syringe in the Luer-opening of the filter holder containing the prefilter and fit it tightly in the opening (*Figure 13*).

7. Flush the total contents of the syringe through the connected filter holders.

8. Repeat steps 5 to 7 with additional volumes of water sample if appropriate (*see remark N.B.b above*).

9. Remove the syringe from the connected filter holders and pull the plunger upwards to fill the syringe with air.

10. Connect the syringe again to the filter holders and push the air through the 2 filter holders to eliminate all remaining water.

4. Box with membrane filters
A small box containing 25 membrane filters of 13 mm diameter and 0.2 µm porosity, for capturing the bacteria during application of the membrane filter test.

5. Box with pre-filters
A small box containing 25 nylon prefilters of 13 mm diameter and 10 µm mesh, to capture all biological material and biological residues which are not of "microbial" origin, during performance of the membrane filter test.

6. Mini filter holders
Two 13 mm filter holders in inert plastic with Luer fitting, to hold a prefilter and a membrane filter respectively.

7. Pincette
A pair of plastic tweezers for the manipulation of the prefilters and the membrane filters.

8. Cleaning stick
A long metal stick provided on one end with a small piece of foam, to clean the measurement compartment of the luminometer in case of spilling of the reagent.

9. Holders for the tubes with ATP reagents
Two transparent plastic holders to be fitted to the tubes with the ATP reagents, prior to insertion of these tubes in the luminometer.

10. Finnpipette 1000 µl
A 1 ml Finnpipette, to be fitted with 1 ml finntips, for application of plate count tests.

11. Finnpipette 200 µl
A 0.2 ml Finnpipette, to be fitted with 200 µl finntips.

12. Timer
A small timer with digital display (*only needed for Toxi-Screening tests*).

Analyses in the field only require transportation of the Luminescence Measurements Case and of one Unit Box, for assessment of the degree of bacterial contamination of 2 water samples.

TRANSFER OF THE MATERIALS FROM A UNIT BOX TO THE LUMINESCENT MEASUREMENTS CASE

On arrival in the field, open the Unit Box and place the 2 tubes with ATP reagents and the 2 finntips in the holes in the upper left corner of the Luminescence Measurements Case *(Figure 3).*

DIRECT TEST

1. With the aid of the plastic pincette, tear open the top cover of one tube with ATP reagents and remove the cover completely (*Figure 4).*

2. Put one of the 2 finntips on the 200 µl Finpipette, take a 200 µl water sample and transfer it into the upper chamber containing the liquid ATP extractant (*Figure 5).*

3. Mix the water sample thoroughly with the ATP extractant by sucking up and pushing back the solution in the upper chamber several times with the Finpipette.

4. After 1 minute, suck up again 200 µl of the mixture, and slowly push the tip of the finntip further down in the tube, through the partition separating the two compartments. Take care during this operation not to spill liquid through the opening of the top compartment of the tube !

5. Empty the contents of the finntip into the bottom chamber which contains the solid ATP reagent. With the same finntip, transfer in several steps all the water/extractant mixture from the upper chamber to the lower chamber.

Figure 10

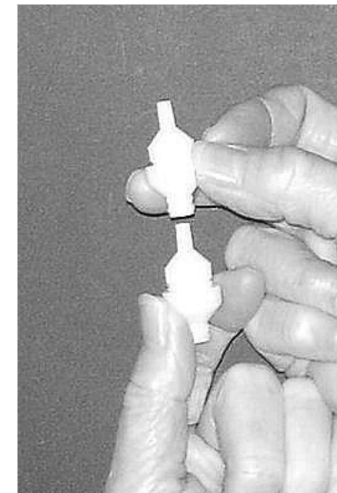

Figure 11

Evaluation of the degree of contamination of water samples by bacteria and biological residues

Although the volume of water analysed by the direct method is quite small (only 200 µl), high RLU scores may nevertheless be noted in case of substantial contamination of the suspect water by microbes and/or biological residues.

A gross ranking of the contamination of the water sample can be made as follows :

< 50 RLU	: very low contamination
< 200 RLU	: low contamination
200-1000 RLU	: significant contamination
> 1000 RLU	: very high contamination

MEMBRANE FILTER TEST

As indicated above, the direct method reflects the "total" amount of ATP in the water sample, without distinction of the ATP from bacteria and that from (solid) biological residues or other (unicellular or multicellular) organisms, nor from the (dissolved) extracellular ATP from dead biota..

A second test procedure can be applied for specific analysis of the ATP from the bacteria present in the suspect sample, after prior elimination of all other (interfering) ATP as well as of dissolved chemicals which can interfere with the luminescence reaction. The membrane filter test procedure captures the bacteria on a small 0.20 µm membrane filter, prior to extraction of their intracellular ATP.

1. Unscrew the 2 parts of the 2 filter holders and place them in the empty holes on the top left side of the Luminescence Measurement Case.

2. With the aid of the plastic pincette, take a prefilter from the small box with prefilters and place it in the hollow compartment of the first filter holder. Reassemble subsequently tightly the 2 parts of this filter holder.

Figure 3

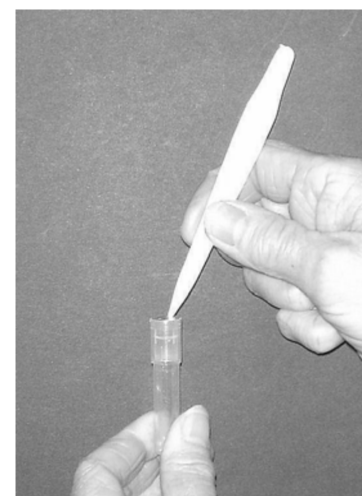

Figure 4

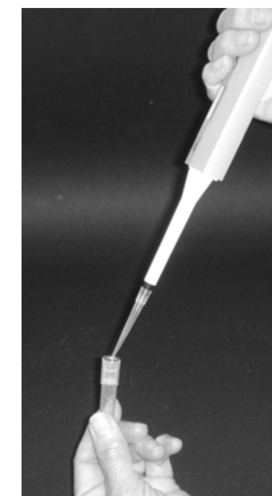

Figure 5

N.B. It is imperative for a reliable measurement that the <u>total volume</u> of water sample + extractant be transferred into the bottom compartment of the tube !

6. Adjust one of the two transparent tube holders tightly to the tube *(Figure 6)* and swirl the holder with the tube gently to mix the water/extractant mixture with the solid ATP reagent till the latter is dissolved completely.

7. Place the tube with the holder in one of the holes of the foam insert layer in the Luminescence Measurement Case.

8. <u>After about 2 minutes</u>, take the luminometer out of the Luminescence Measurement Case, open the lid and insert the holder with the tube (*Figure 7*).

9. Close the luminometer (*Figure 8*) and push the "Power" button. The instrument will calibrate itself by a 10 seconds countdown visible on the display, followed by a beep signal.

N.B. The luminometer must always be kept vertically when containing a holder and a tube !!

10. Push the "Enter" button; the luminometer will again calibrate itself for 10 seconds, after which the luminescence score will appear on the display (*Figure 9*). Note the RLU score on the Results Sheet.

<u>Important remark</u> : the time span between the transfer of the liquid from the top compartment to the bottom compartment of the tube, and the scoring of the luminescence, should be kept (as close as possible) <u>to 2 minutes</u>. Shorter or longer contact times of the dissolved ATP with the ATP reagent will indeed give lower luminescence scorings.

11. Remove the holder with the tube from the luminometer and switch off the instrument by pushing the "power" button.

N.B. When a tube with its holder is left for more than 15 seconds in the luminometer after the scoring, the instrument will give beep signals indicating that the holder and the tube must be removed.

A second water sample can be analysed the same way with the second tube with ATP reagents and the second finntip of a Unit Box.

Figure 6

Figure 7

Figure 8

Figure 9