Manual for Marine Monitoring in the

COMBINE

Programme of HELCOM

Part C

Programme for monitoring of eutrophication and its effects

Annex C-11

Guidelines concerning
bacterioplankton growth determination

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ANNEX C-11: GUIDELINES CONCERNING BACTERIOPLANKTON GROWTH DETERMINATION

1. INTRODUCTION

Bacterioplankton growth rate is an indicator of the nutrient status in aquatic environments. It is an estimate of the consumption of organic carbon in the ecosystem and therefore closely related to the biochemical oxygen demand *in situ* (cf. BOD_7). Bacterioplankton growth rate thereby indicates the rate of oxygen consumption that may lead to oxygen deficiency in the water column when exceeding oxygen supply. The growth rate indicator may be used in all aquatic environments.

The original method is published in international scientific journals and has been used in many marine research studies since the beginning of the 1980´s. The method has been part of the Helsinki commission guidelines for a longer period of time (Baltic Sea Environment Proceedings No. 27D). The current protocol is an adaptation from Smith and Azam [1].

Bacterial growth rate is a relatively unambiguous indicator of the flux of organic matter through the pelagic ecosystem [2]. Even if the relationship between the factors specific growth rate and abundance may vary, their product representing growth rate reflects the substrate supply of organic matter to the bacterioplankton community. Density limitation (i.e. competition) or other limiting factors (i.e. inorganic nutrients, temperature) do not therefore directly appear to control the bacterioplankton community growth rate at typical environmental conditions. This agrees with empirical observations that bacterial growth rate over larger scales correlates with trophic status of a system [2, 3], and at smaller scales between water layers and seasons [4]. This is true for a growth rate range covering several orders of magnitude.

Bacterioplankton growth rate may be complemented by bacterial abundance and biovolume estimate providing better precision in the biomass production values. This also allows a deeper understanding of whether specific growth rate or bacterial abundance explains changes in community growth rate.

1.1 BACKGROUND

Bacterioplankton are osmotrophs feeding on dissolved organic carbon and dissolved mineral nutrients. They often live like solitary cells free floating, but may also grow attached to particle surfaces. Bacterioplankton typically divide by binary fission, are rod shaped, spherical or c-shaped with an average dimension of 0.6 µm. Small heterotrophic flagellates are their main predators.

The rate of bacterial biomass production was suggested as an indicator of the consumption of organic carbon in an ecosystem by Billen et al. [2]. A positive relationship between nutrient status and bacterial growth, as well as biomass, across different ecosystems has been demonstrated in independent studies [2, 3]. Increased organic production is detected by the variable, whether due to increased phytoplankton growth or import of organic matter from river and waste water discharge.

Bacterial biomass production is closely linked to biological oxygen demand in an environment, and bacterioplankton accounts for about 50% of the oxygen demand in aquatic environments [5]. This is due to oxygen constituting the major electron acceptors in aerobic environments and that bacteria channel a large part of the carbon flux in aquatic ecosystems. Bacterioplankton respiration is therefore an important cause of oxygen depletion when eutrophication prevails, as more than 80% of the marine secondary production occurs in the pelagic environment [6, 7].

1.2 PRINCIPLE

Bacterial growth rate is estimated by uptake of the DNA base thymidine that is radioactively labelled. Thymidine has been shown to be almost exclusively taken up by heterotrophic bacteria in natural samples [1, 8]. Uptake by photosynthetic plankton does not seem to interfere significantly. The synthesis of DNA in a cell is coupled to cell division. Before a cell may divide, the DNA should have doubled to provide all genetic information required for the cell. Thereby twice as much thymidine should have been incorporated when the cell is ready for division.

Thymidine labelled with tritium (^{3}H) in the methyl group is used. The amount of thymidin taken up is thereby proportional to the amount of radioactivity taken up.

The amount of thymidine taken up is transformed to the number of cells produced by empirical knowledge of the amount of thymidine per cell on average. The theoretical conversion factor correlates relatively well with the empirically derived, but is typically slightly below the latter. The reason is that thymidine pools within the cell dilute the added radioactive thymidine, leading to some underestimation of the true thymidine incorporation by theoretical factors. The fact that some bacteria do not assimilate thymidine, and that some predation on bacteria occur during incubation, also leads to somewhat higher empirical conversion factors.

Bacterial cell growth may be transformed to biomass production by knowing the carbon content per cell. Further transformation to oxygen consumption can be used by using literature values of growth efficiency and respiration quotient (RQ).

1.3 EXTENT

Bacterial growth rate may be applied in freshwater as well as oceanic salinity (0-35). The requirement is that radioactive thymidin is added in sufficient excess to natural extra-cellular pools (e.g. 25 nmol dm^{-3} tritiated thymidine in brackish water). The applied conversion factor should also be valid for the studied environment.

1.4 DISTURBANCES

Avoid exposing the sample to markedly different temperature or light irradiance compared to *in situ* conditions. Ice-cold (0°C) TCA solutions and tubes are essential for the precipitation step. Be careful to pre-cool solutions and tubes prior to use.

Careful removal of the supernatant after centrifugation is a critical step in the procedure. The sample tubes should be kept at room temperature during this procedure to avoid formation of mist on the tube walls. Use a glass pipette (Pasteur) where the tip has been narrowed by heating over a gas burner. Remove all liquid to the bottom, following the side opposite to where the precipitate is expected. Also remove any drops under the lid.

Do not use latex protection gloves as they may create fluorescence.

1.5 CONTAMINATION RISK

Work antiseptically with sterile tips and tubes. Avoid especially contaminating the thymidine stock solution by carefully removing aliquots for each experiment to a sterile tube.

Keep the samples away from any biocide (e.g. formaldehyde, Lugol solution, Latex rubber, TCA etc.).

1.6 SAFETY

ISOTOPE

Tritiated [metyl-3H] thymidine with a specific activity of typically 80 000 Ci mol^{-1} and concentration of 12.5 µmol dm^{-3} (1mCi/ml) is used. The isotope is a ß-emitter and has a range of 10 mm in water. Protect face and eyes from concentrated stock solution. Use a laboratory coat and protective gloves. Diluted working solution should be handled according to laboratory procedures.

TCA

TCA is corrosive on eyes, skin and mucous membrane. Vapour and dust may cause irritation and harm lungs. Use mouth protection, protective gloves and laboratory coat when weighting the substance. Work in a ventilated hood.

2. PREPARATIONS

2.1 CLEANING AND PURIFICATION

Use tubes well rinsed with Milli-Q water for sub-samples the sampling bottles, or taken directly from the bag. These tubes may be re-used following rinsing with Milli-Q water. All bottles and tubes should be clean and not have been in contact with biocides like TCA or formaldehyde.

Tips for e.g. automatic pipettes may be re-used following rinsing with Milli-Q water. Only use tips exclusively for each solution.

Rinse the tip with Milli-Q-water between each depth when dispensing water samples.

2.2 IDENTIFICATION OF SAMPLE

50 ml polypropylene tubes for sub-sampling should be labelled with variable, sample depth and replicate (if applied).

Micro-centrifuge tubes (1.5 ml) are labelled with cruise, station, depth and treatment on the lid with water resistant marker pen.

Place samples in proper order to simplify data treatment.

2.3 REAGENTS

ISOTOPE

Tritiated [methyl^{-3}H] thymidine according to item p. 9 is used.

TRICHLOROACETIC ACID (TCA)

TCA contains a lot of crystal water. 100 % TCA is prepared by mixing 500 g TCA (e.g. Merck, ProAnalysi) with 227 l Milli-Q water. 5 % and 50 % TCA is prepared from the concentrated solution by dilution with Milli-Q water.

TCA is corrosive for eyes, skin and mucous membrane. Vapour and dust may be irritating and cause lung damage. Use mouth protection and laboratory coat. Work in a ventilated hood.

SCINTILLATION LIQUID

Touluene- and Xylene free scintillation liquid is recommended (e.g. Optiphase HiSafe, Wallac OY). The scintillation liquid should be possible to mix with water.

ICE

Crushed ice may be used as cooling medium for TCA tubes.

2.4 BEFORE CRUISE/SAMPLING

Eppendorf tubes (1.5 ml) are placed in 5 ml scintillation vial without lid. The tubes are labelled with cruise, station, depth and treatment. Place the tubes in the order that results are wanted to appear in the scintillation file or print out.

At the beginning of the sampling day incubator for tubes and cold centrifuge are switched on for pre-cooling to the desired temperature. Label tubes for sub-sampling (i.e. 50 ml Falcon tubes) with station and depth, and place them in racks. Have two thermoses ready labelled “Above thermocline” and “Below thermocline”, respectively. Have a glass pipette (Pasteur-type) with thin tip attached to a vacuum source (e.g. water tap vacuum device). Bench surfaces used with radioactive samples should be covered with protective paper.

2.5 PROTOCOL

A sampling protocol for logistic data according to ICES recommendation should be used.

3. SAMPLING

3.1 SAMPLING

3.1.1 SAMPLING STRATEGY

It is recommended to take at least 2 samples at different representative depths of the monitored layers of the water column. Surface layer and deepest layer are prioritised. Layers are defined by hydrographic profiles. Required power of the data and natural variability set the required number of samples.

A sampling frequency of 10 samples per year is required to get confident annual estimates (J. Wikner, unpubl. results). Samples should be distributed in the seasonal curve to provide a good coverage of different levels (more samples during the productive season).

An economic alternative is to allocate at least 2 samples to a representative month with limited inter-annual variation. This strategy, however, results in a lower power to detect trends and less ability to cover changes in seasonality. August is recommended based on current experience. Low frequency stations should preferably be evaluated together with high frequency stations located in the same sea area.

It is advocated that at least one high frequency station of 18 samples per year is monitored in each contracting country. This allows an analysis of intra-annual variation and for following changes in seasonal dynamics.

3.1.2 SAMPLING METHOD

Sampling may be performed with a rosette sampler or Niskin bottles attached to a wire.

Water samples are collected according to HELCOM guidelines.

A Milli-Q rinsed polypropylene tube is rinsed once with sample water before a sample of 50 ml is collected. Store the tube with a closed lid as close to *in situ* temperature as possible until start of the incubation according to item 4.4.1.

Fill the thermoses with water from the surface and deep water layer to be used for incubation of samples from depth with similar temperature.

3.2 PRESERVATION/PROCESSING

Processing is done within 1 hour from sampling according to item 3.3 and 4. At rough weather processing may wait up to 8 hours. Note delays exceeding 1 hour in the protocol.

3.3 STORAGE

Tubes with sub-samples are stored as close to in *situ* temperature as possible. Refrigerator or other incubators may be used.

Samples in micro-centrifuge tubes with 50% TCA added may be stored at 4°C for up to 7 days before processing.

Micro-centrifuge tubes with TCA precipitated material in scintillation liquid may be stored at room temperature and in the dark until analysis in a scintillation counter. Counting should be done within 5 days.

4. METHOD DESCRIPTION

4.1 REAGENTS

Make 50 % (w/v) TCA and 5 % (w/v) TCA in sufficient volume to last at least one cruise. Store working solutions of TCA in polypropylene tubes (e.g. Falcon®) submerged in ice slurry during the whole processing procedure.

Withdraw the volume of [methyl-^{3}H] thymidine that is required to run analysis at one station to a sterile (fresh) Micro-centrifuge tube.

4.2 CALIBRATION SOLUTIONS

None.

4.3 PROCESSING

4.3.1 PREPARATIONS

Switch on the cold centrifuge to pre-cool the rotor to +4°C. Centrifuges without cooling may be put in a refrigerator. If an incubator like micro-centrifuge tube ThermoStat plus is used, it is set to +2°C. If a cold plexiglass block is used, it should be tempered at least one hour before use at -20°C.

4.3.2 UPTAKE OF LABELLED THYMIDINE

- For every sample depth two Micro-centrifuge tubes are filled with 1 ml of sample. Additional replication per depth may be applied as appropriate. Replicates should be measured on at least one station per cruise for analytical quality assurance. The same tip may be used for all depths provided that rinsing with two sample volumes Milli-Q water is done in between
- Add 100 ml 50 % TCA to the background treatment samples, mix 3 s with a blender and incubate for 5 min. TCA stop cell activity in the samples
- Withdraw the amount of [^{3}H-methyl] thymidine that is required for one station from the stock solution to a clean micro-centrifuge tube. Add 2 µl of thymidine to samples and then background samples. The same tip may be used for all tubes, by placing the drop of isotope on the wall above the water surface in the sample tube. Mix the tubes 3 s in a blender.
- Place the tubes in the thermos with closest temperature to the water depth of the sample. Note time of incubation start, specific activity and batch number in the protocol. Incubate for 1 hour. If a cooled plastic block is used, cool it at -20 °C in the mean time
- Stop the incubation by placing the micro-centrifuge tubes in the cooling device used at +2 °C for 5 min. Note the stop time in the protocol

4.3.3 PRECIPITATION OF BACTERIAL BIOMASS WITH TCA

- Add 100 ml 50 % TCA to the samples (*not* the background vials) and mix for 3 s. The TCA solutions should be ice-cold at this step. Incubate the samples at +2 °C or on ice for 5 min. If centrifugation can't be done directly, samples may be stored in this condition at +4 °C for up to 7 days
- Place the micro-centrifuge tubes in a cooled (+4 °C) centrifuge with the "necks" facing outwards and towards the rotor. Samples should not be frozen at this stage. Centrifuge the micro-centrifuge tubes at 16000 x g (13000 rpm, see item 8 Equipment) for 10 min. If not all tubes fit in the rotor, store the remaining tubes in the refrigerator
- Place the micro-centrifuge tubes in a tube rack at room temperature. Remove the supernatant with a Pasteur pipette with a thin tip using a vacuum source. Note that the supernatant is radioactive. Be very careful to remove all liquid. Also remove all mist and droplets on the tube wall and under the lid. The typically invisible pellet is located in the tube bottom facing outward from the rotor. Some precipitate may however stick to the tube wall on the same side. Don't touch the pellet
- Wash the pellet and tube with 5 % TCA. Make sure that no air bubbles are left in the bottom of the tube so that the pellet is washed properly. Close the lid, mix the sample 5 s and turn it up-side down to also wash the inside of the lid
- Centrifuge the micro-centrifuge tubes with the "necks" facing outward at 16000 x g (13000 rpm) for 10 min. Remove the supernatant as above
- Add 1 ml of scintillation liquid to each tube. Close the lid and mix 5 s on a blender. Hang thee micro-centrifuge tubes in the 5 ml scintillation vials. Store the samples as defined in item 3.3.

4.4 CALIBRATION

The scintillation should be calibrated with sealed standards, typically provided by the manufacturer. Record calibration date and result. Change standards before the expiration date.

Quench correction curve installed by the manufacturer is typically used.

4.5 ANALYSIS

Scintillation counting can be done in the 5 ml scintillation vials with micro-centrifuge tubes.

Run standards before the samples and follow the manual of the scintillation counter.

Record the results preferably in a computer file to minimize errors while entering values manually and save time. Data may also be printed.

The samples are counted in the [^{3}H]-window and settings generating disintegrations per minute (DPM) values from counts per minute (CPM) based on a quench curve installed by the manufacturer (see item 4.4).

The following settings have successfully been used in a Beckman LS6500 scintillation counter for counting tritium:

ID: 3H, 5MIN, DPM

User : 1 Comment:

Preset time : 5.00

Data calc : SL DPM H#: : Yes Sample repeats : 1 Printer: : STD
Count blank : No IC# : no Replicates : 1 RS232 : OFF
Two phase : No AQC : no Cycle repeats : 1
Scintillator : liquid Lumex : no low sample rej: 0
Low level : no Halflife correction date: none

Isotpe 1: 3h %error: 2.00 Factor: 1.000000 BKG.SUB:0

Background quench curve: Off Color quench correction: On

Quench Limits Low: 2.672 High: 316.80

5. CALCULATIONS

5.1 CALCULATION FUNCTIONS

5.1.1 TRANSFORMATION OF DPM TO CELL GROWTH

The amount (mol) of incorporated ^{3}H-thymidine ml^{-1} h^{-1} (n_{ty}) is calculated as:

$$\Delta n_{ty} = \frac{(dpm_s - dpm_b) \times 4.5 \times 10^{-13}}{v \times \Delta t \times SA} \quad (1)$$

where

dpm_s=disintegration per minute in the sample (average of replicates if present)

dpm_b= disintegration per minute in the background (average of replicates if present)

4.5*10^{-13}=conversion factor (dpm ==> Ci)

v= sample volume (cm^3)

Δt= incubation time (hours)

SA= specific activity for [^{3}H]-thymidine (Ci mol^{-1})

Bacterial growth in cells (P_c) is calculated as

$$P_c = \Delta n_{ty} \times TCF \times 24 \times 1000 \quad (2)$$

where *TCF* is the thymidine cell conversion factor. A conversion factor empirically determined for the Baltic Sea area of 1.4 x 10^{18} cells [mol thymidine]$^{-1}$ (n=73, ±SE=0,1 x 10^{18}) is recommended. This factor seems independent of growth rate and is close to the theoretical factor for coastal environments [4, 8-12].

The factors 24 and 1000 transform cells cm^{-3} h^{-1} to cells dm^{-3} day^{-1}.

5.1.2 BACTERIAL BIOMASS PRODUCTION

Cell production is transformed to bacterial biomass production (P_b, mol carbon dm^{-3} day^{-1}) with the function

$$P_b = P_c \times m_b \quad (3)$$

The factor m_b is the carbon content of cells on average in the sample in µmol C cell^{-1}. See the standard operating procedure for bacterioplankton biomass for a definition.

5.1.3 BACTERIAL OXYGEN CONSUMPTION

Bacteral oxygen consumption, ΔO_2^{bact}, may be calculated from P_b , bacterial growth efficiency, *BGE*, and the respiration quotient, *RQ*, according to:

$$\Delta O_2^{bact} = P_b \times \frac{1 - BGE}{BGE} \times RQ \quad (4)$$

Estimates of BGE are currently uncertain and vary with at least nutrient status. Recalculation of bacterial growth to bacterial oxygen consumption is therefore a crude estimate of the latter. The best estimate of BGE is probably obtained by the function reported by Del Giorgio and Cole, 1998 [13]

$$BGE = \frac{0.037 + 0.65 \times P_b}{1.8 + P_b} \quad (5)$$

where *Pb* is the bacterial growth rate in µg C dm^{-3} h^{-1}. The bacterial growth efficiency average 0.27 in the reported data set, which is close to constants used in the literature. The uncertainty of the function has not been reported.

Values of 0.9 has been used for the respiration quotient (RQ), based on a weighted average for respiration of carbohydrate (weight 0.5), protein (weight 0.33) and fatty acids (weight 0.17) [14]. This RQ is also in good accordance with results from experiments on a marine bacterium [15].

5.1.4 STANDARD DEVIATION

The standard deviation (SD_{tot}) for replicates at one sample depth is calculates as the square sum of both sample and background treatments according to:

$$SD_{tot} = \sqrt{\left(SD_s^2 + SD_b^2\right)} \quad (6)$$

where SD_s och SD_b are the standard deviation for samples and backgrounds, respectively.

5.1.5 THE VARIATIONS COEFFICIENT

The variation coefficient (CV_{tot}) is calculated as:

$$CV_{tot} = \frac{SD_{tot}}{\overline{m}} \quad (7)$$

where m is the average netto dpm based on the difference between samples and background.

5.2 CALCULATIONS

Values for assimilated thymidin (dpm), background and other factors are entered in a database or calculation software according to table 2. Calculation functions according to item 5.1 are applied.

The calculation should return parameters and units according to Table 3 in item 7.

5.3 MEASUREMENT UNCERTAINTY

The measurement uncertainty has been determined according to the standard of measurement uncertainty in chemical analysis of the European Union [16].

The standard uncertainty corresponds to standard deviation and is estimated from several identified variance components of the method. The assimilation of thymidine shows a low expanded uncertainty of ±21%, approximately corresponding to a 95% confidence interval (Table 1). Conversion factors contribute with the greatest uncertainty.

Table 1: Measurement uncertainty for bacterial growth rate. U is the expanded uncertainty with a factor 2.

Parameter	Unit	Value	U (%)
Bacterial growth	µmol C dm^{-3} day^{-1}	0.29	±21

The coefficient of variation (±CV) for netto dpm should stay below 20% in productive waters. During the winter season values may be somewhat higher. Values above ±60 % should be scrutinized.

Background values should stay below 100 dpm and average 30 dpm.

The detection limit corresponds to 100 dpm netto uptake of thymidine (+2 x SD). This corresponds to 1 x 10^7 cells dm^{-3} day^{-1} or 0.02 µmol dm^{-3} day^{-1}, approximately the same in carbon or O_2. Typical growth rates in mesotrophic environments are 20 times higher.

6. QUALITY ASSURANCE AND EVALUATION

6.1 CONTROL CHARTS

Duplicate samples should be run regularly corresponding to about 10% of the samples. Plot the standard deviation of duplicates against date in a control chart.

Background values are plotted in control chart.

6.2 EVALUATION

For evaluation of all charts use alarm (2 x SD) and action limits (3 x SD). Values above the action limit should be evaluated for potential error sources. If errors are found, they are corrected with date, motivation and signature added. If no error can be identified, values are labelled as extreme values or questionable values.

It's recommended plotting a full year of data at the end of the year of measurement, to get a good view of the seasonality and depth variation. Sample dpm and growth rate parameters may be plotted against date and depth. Values should be plausible and not differ more than 3 x SD from the average values during a given season. Values should also show an expected variation with depth, where surface values typically are greater than those in deeper water.

Correct found errors and note date, motivate change and sign the change made. Label deviating values as extreme or questionable values if no errors can be identified. Avoid deleting values without proper reason.

7. REPORTING

Enter data in a data base or calculation program as described in Table 2, together with logistic data to identify the sample according to recommendation by the International Council for Exploration of the Sea (ICES).

Table 2: Primary database variables and units.

Parameter	Unit	Digits	Function	Category	Acronyme	Value ex..
Sample radioactivity	dpm	3	-	Depth	BGSAMDPM	1000

Parameter	Unit	Digits	Function	Category	Acronyme	Value ex..
Background radioactivity	dpm	3	-	Depth	BGBKGDPM	40
Specific activity	Ci mol^{-1}	3	-	Depth	BGSPACTY	82000
TCF†	cells mol^{-1}	3	-	Depth	BGTCF	$1.4x10^{18}$
Sample volume	cm^{-3}	3	-	Depth	BGSAMVOL	1
Start of incubation	tt.mm	4	-	Depth	BGINCST	10.18
End of incubation	tt.mm	4	-	Depth	BGINCEN	11.2
Respiration quotient	-	2	-	Depth	BGRQ	0.9
Bact. growth efficiency	%	2	5	Depth	BGGREFF	0.3
Date of calibration (scint.)	01-09-2026	6	-	Depth, standard	BGICD	01-10-2004

† Thymidin conversions factor transforming uptake of thymidine in mol to cells produced.

Table 3: Calculated parameters of bacterial growth rate.

Parameter	Unit	Digits	Function	Category	Acronym	Value ex.
Thymidine uptake rate	mol cm^{-3} h^{-1}	3	5.1.1.	Depth	BGTHYUP	5.11´10-15
Thym. uptake rate ±SD	mol cm^{-3} h^{-1}	2	5.1.1, 5.1.5	Depth	BGTHYSD	$4.13x10^{-16}$
Bacterial cell production	cells dm^{-3} day^{-1}	3	5.1.1	Depth	BGCELLP	$1.84x10^{8}$
Bact. Prod. ±SD	cells dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCCELSD	$1.12x10^{7}$
Bact. Carbon production	µmol C dm^{-3} day^{-1}	3	5.1.2	Depth	BGCARPR	0.29
Bact. Carbon prod. ±SD	µmol C dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCARCV	0.02

Parameter	Unit	Digits	Function	Category	Acronym	Value ex.
Bact. oxygen consump.	µmol dm^{-3} day^{-1}	3	5.1.3	Depth	BGCOXYCO	0.6
Bact. oxygen ±SD	µmol dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCOXYSD	0.05

Use quality codes according to ICES directives.

Use ICES format when reporting logistic information with each value.

Valuable variables of explanation include bacterial biomass (whole community, µmol C dm^{-3}), bacterial volume (median, μm^3 $cell^{-1}$), bactivorous flagellates (flagellates dm^{-3}), temperature (°C), total phosphorus (µmol dm^{-3}), total nitrogen (µmol dm^{-3}) and oxygen (µmol dm^{-3}). Substrate variables may also be used if available, where total DOC is a crude indicator of substrate availability.

8. EQUIPMENT

PLASTIC- AND GLASS WARE

- Polypropylene tubes (50 ml) with lid (e.g. Falcon®)
- 1.5 ml micro-centrifuge tubes of polypropylene (e.g. Eppendorf®)
- Scintillation vials (6 ml). (e.g. Beckman Mini Poly-Q-vial)
- Pipette tips 0.5-10 µl, 10-100 µl, and 100-1000 µl
- Pasteur pipettes of glass with a narrow tip. Narrow the tip by melting the pipette over a gas burner, gently pulling each end of the pipette apart. Break the pipette at the narrowest position.

REFRIGERATED CENTRIFUGE

A refrigerated centrifuge for micro-centrifuge tubes (1.5 ml) that can achieve the desired g-force and 4 °C is required. One example is a Beckman GS-15R with rotor F2402. Centrifuges that do not manage refrigeration may be run inside a refrigerator. The rotor should be chilled before applying the samples.

WATER VACUUM DEVICE

A vacuum pump with capacity of at least -400 mmHg is required. A Pasteur pipette with narrowed tip is connected to a water vacuum device attached to a regular tap to remove the supernatant. This also discards the radioactive liquid directly into the sink. Alternatively a water trap may be installed between the vacuum source and the Pasteur pipette.

AUTOMATIC PIPETTES

Calibrated pipettes covering volume ranges of 0.5-10 µl, 10-100 µl and 100-1000 µl is required. A motor driven pipette is recommended for dispensing liquid to many samples.

REFRIGERATED INCUBATOR

A refrigerated incubator with room for at least 14 micro-centrifuge tubes is recommended to use (e.g. Eppendorf ThermoStat plus, prod nr. 5352 000.010 + 5364 000.016). A temperature of +2°C has been found to give an optimal precipitation of cell material by TCA. The incubator should be pre-chilled for 30 min.

COOLING RACK

A solid cooling rack (e.g. plastic) with holes for 1.5 ml micro-centrifuge tubes may be used to chill samples, as an alternative to a refrigerated incubator. The rack should be chilled at -20°C. The rack keeps sufficient cooling capacity for 15 min. at room temperature. Store the rack in the freezer when not in use.

SCINTILLATION COUNTER

A scintillation counter with internal quench correction is recommended. One example is Beckman Coulter™ LS 6500 Multi-Purpose Scintillation Counter (cat.nr. 510656). Beckman's™ software for digital collection of data to a computer file is used.

TUBE BLENDER

A laboratory blender for tubes is recommended. One example is Vibrofix VF1, Jankel & Kunkel, IKA-Labortechnik.

9. CHEMICALS AND SOLUTIONS

ISOTOPE

A fresh solution of tritiated thymidine less than 8 weeks old from activity date should be used. Make sure the isotope has the desired specific activity (about 80 000 Ci mol^{-1}) Note the date of arrival and volume used on the vial. Store the isotope in the refrigerator.

Tritiate [methyl-^{3}H] thymidine (e. g. Amersham order no. TRK 686) with specific activity of 80 000 Ci mol^{-1} and concentration of 12.5 µmol dm^{-3} (1mCi ml^{-1}) is used. Withdraw the volume required for one station to a clean micro-centrifuge tube to minimize the risk of contamination. Use sterile tips with the pipettes.

The [methyl-^{3}H] thymidine is a ß-emitter where electrons have a range of about 10 mm in water. Minimise handling of the concentrated stock solution. Use protective gloves and a laboratory coat. Discard the diluted isotope according to local regulations.

TRICHLOROACETIC ACID (TCA)

Trichloroacetic acid contains large amounts of crystal water. 100% (w/v) of TCA is made by mixing 500 g of TCA powder (e.g. Merck, ProAnalysi) with 227 cm^3 of Milli-Q water. Diluted solution of 50 % and 5 % are made from the stock solution by dilution with Milli-Q water.

TCA is corrosive on eyes, skin and mucous membrane. Vapour and dust may cause irritation and damage on the lungs. Use mouth protection and protective gloves and a laboratory coat. Work in a ventilated hood with TCA powder.

SCINTILLATION LIQUID

Toluene- and Xylen free scintillation liquid is recommended (e.g. Pharmacia OptiPhase HiSafe 3, Wallac OY, prod. no. 1200-437).The scintillation liquid should be possible to mix with water.

MILLI-Q WATER

Milli-Q water is made from deionized water that is further purified through an ion exchange resin and 0.2 µm filter. Devices producing Milli-Q water is manufactured by e.g. the Millipore ® Company.

ICE

Crushed ice may be use as a cooling medium for tubes with TCA.

10. REFERENCES

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