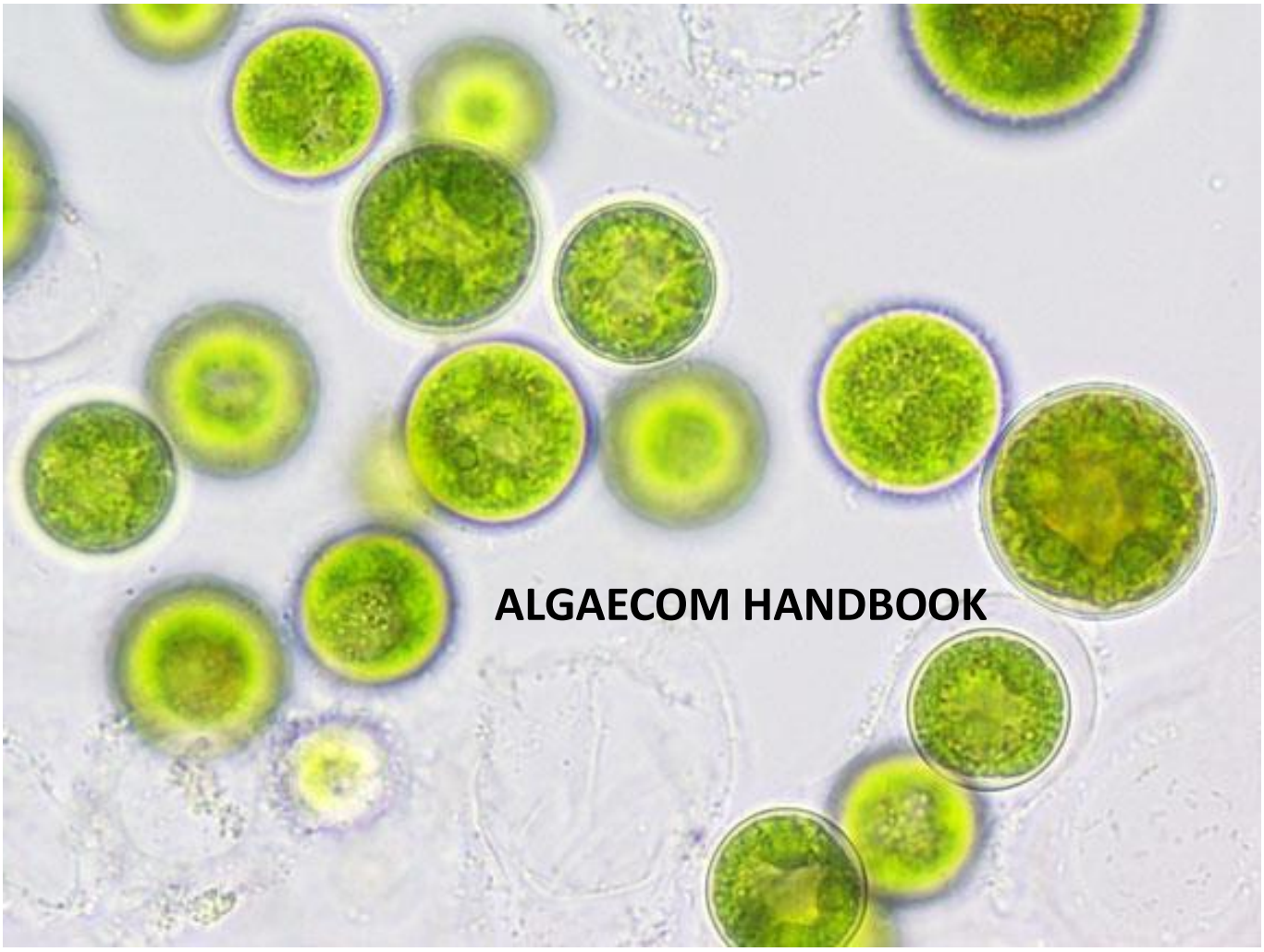

ALGAECOM HANDBOOK

Athens, 2015

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1. Superoxide Dismutase (SOD) Assay

a. WST-based assay

This assay relies on the reduction of the dye WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H- tetrazolium, monosodium salt) with a superoxide anion according to the following scheme:

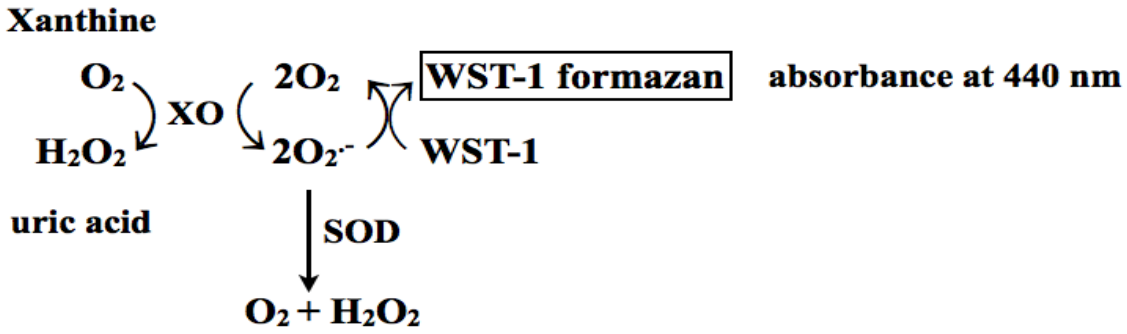


Figure 1.

The rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity, and is in turn, inhibited by SOD, as shown in the Figure 1 above. Subsequently, the IC50 (50% inhibition activity of SOD or SOD-like materials) can be determined as a decrease of the absorbance at 440 nm which is the absorption maximum of WST-1 and is proportional to the amount of superoxide anion. This method necessitates the generation of an initial inhibition reference curve with known SOD activities from which the activity of the unknown sample will be calculated.

<u>Solutions</u>

a) WST Solution
b) Enzyme Solution (Xanthine Oxidase; XOD)
c) Buffer Solution (Xanthine)
d) Dilution Buffer (buffer of preference)

<u>Equipment required</u>

a) Plate reader with a 450 nm filter

b) 96-well microplate

c) 10 µL & 100-200 µL pipettes and a multi-channel pipette

d) Incubator

e) Superoxide dismutase (SOD) with known specific activity for the preparation of an inhibition reference curve

Preparation of working solutions

a) WST working solution: Dilute 1 ml of WST Solution with 19 ml of Buffer Solution.

b) Enzyme working solution: Centrifuge the Enzyme Solution tube for 5 sec. Mix by pipeting, and dilute 15 µl of Enzyme Solution with 2.5 ml of Dilution Buffer.

c) SOD Solution: Dilute SOD with Dilution Buffer to prepare SOD Standard Solution as follows: 200 U/ml, 100 U/ml, 50 U/ml, 20 U/ml, 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml, 0.01 U/ml, 0.001 U/ml

Procedure

The following solution amounts are used for setting up both the sample and the standard SOD activities.

1) Add 20 µl of sample solution to each sample and blank 2 well, and add 20 µl of ddH_2O (double distilled water) to each blank 1 and blank 3 well.
2) Add 200 µl of WST Working Solution to each well, and mix.
3) Add 20 µl of Dilution Buffer to each blank 2 and blank 3 well.
4) Add 20 µl of Enzyme Working Solution to each sample and blank 1 well, and then mix thoroughly*.
5) Incubate the plate at 37 °C for 20 min.
6) Read the absorbance at 450 nm using a microplate reader.
7) The SOD activity (inhibition rate %) is calculated using the following equation:

SOD activity (inhibition rate %) = {[(Ablank 1 - Ablank 3) – (Asample - Ablank 2)]/ (Ablank 1 - Ablank 3)} x 100

Unit definition: One unit will inhibit the reduction of WST-1 by 50% under the assay conditions described in the present protocol.

Table 1. Amount of each solution for sample (or standard), blank 1, 2 and 3.

	Sample	Blank 1	Blank 2	Blank 3
Sample solution	20 µL		20 µL	
ddH_2O		20 µL		20 µL
WST working solution	200 µL	200 µL	200 µL	200 µL
Enzyme working solution	20 µL	20 µL		
Dilution buffer			20 µL	20 µL

Blank 1: it corresponds to the totally uninhibited reaction

Blank 2: it gives the background reaction of the sample without containing Xanthine Oxidase

Blank 3: it shows the background absorbance of the WST-1

The amounts shown above are suitable when the measurements are done using a microplate reader. Yet, the assay can be scaled up to bigger volumes depending on the availability of the equipment at each lab.

A typical inhibition reference curve is shown in Figure 3, which is the result of the SOD inhibition rates using upscaled volumes of 1 mL in Figure 2 below.

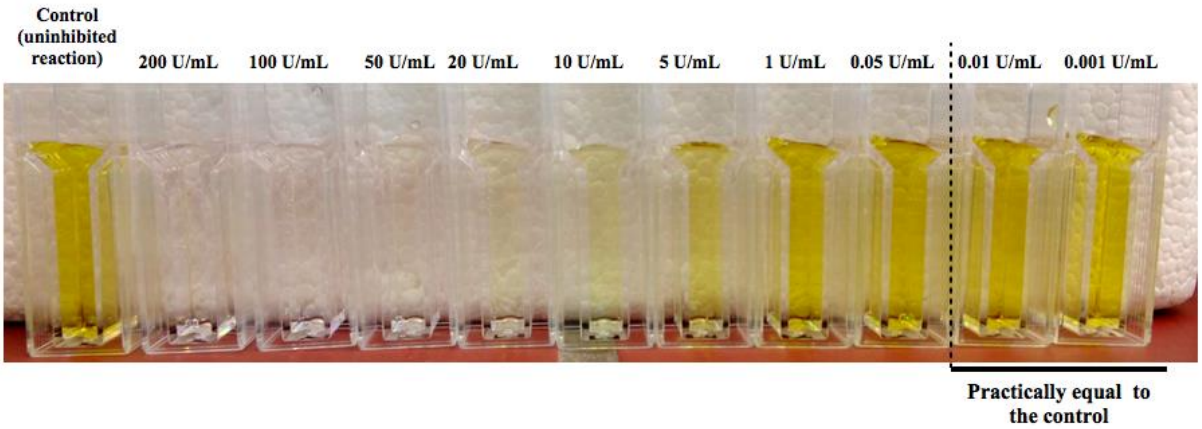


Figure 2.

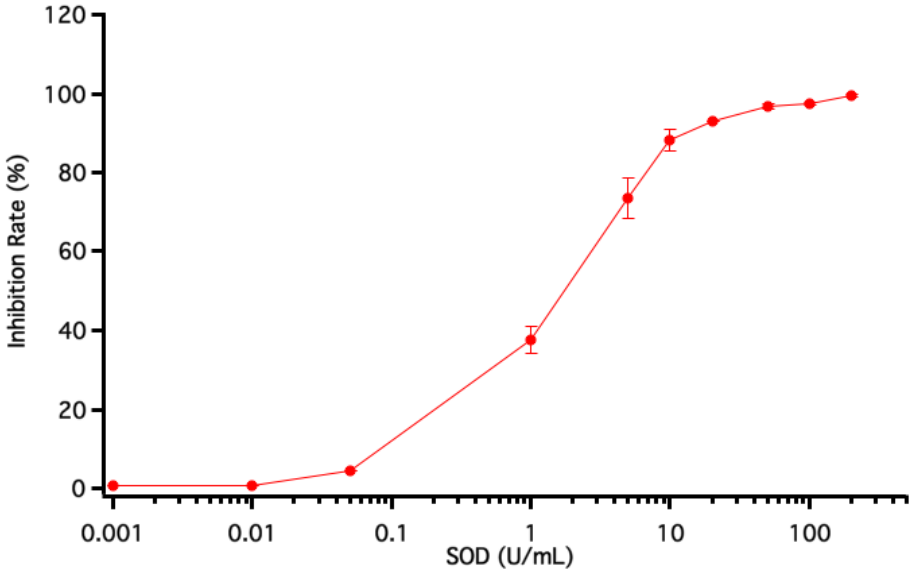


Figure 3.

Notes

1) For more accurate measurements, the use of multiple wells per sample is recommended
2) Superoxide will be released immediately after the addition of Enzyme Working Solution to a well since XOD will start converting its substrate xanthine, the use a multichannel pipette to avoid the reaction time lag of each well is highly recommended.
3) Inhibition activity can also be determined by a continuous kinetic method. A good linearity should be observed up to 20 min.

Storage

WST Working Solution can be stored for 2 months at 4°C. Enzyme Working Solution is stable for 3 weeks at 4°C. WST Solution and WST Working Solution should be protected from light.

b. Cytochrome c-based assay

This assay is based on the same principle as the WST-1 assay described above. However, it makes use of oxidized cytochrome c instead of WST-1. The oxidized cytochrome c is reduced

by the superoxide radical and the rate of reduction is followed spectrophotometrically and continuously at 550 nm as shown in the following Figure:

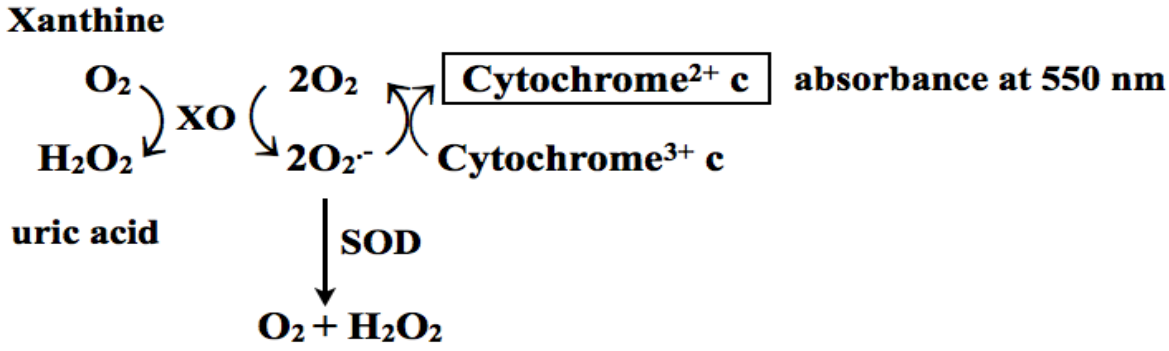


Figure.

Similar to the WST-1 assay, the SOD activity is expressed as the % inhibition rate of the oxidized cytochrome c reduction.

Solutions

a) 216 mM Potassium Phosphate Buffer, pH 7.8 at 25°C: 49.3 mg/ml solution of potassium phosphate dibasic trihydrate in ddH_2O and adjust the pH to 7.8 at 25°C with 1 M KOH or 1 M HCl).

b) 10.7 mM Ethylenediaminetetraacetic Acid Solution (EDTA): 4.0 mg/ml solution of Ethylenediaminetetraacetic acid disodium salt dihydrate in ddH_2O.

c) 1.1 mM Cytochrome C Solution (Cyt C): 14.6 mg/ml solution of Cytochrome C, in ddH_2O.

d) 0.108 mM Xanthine Solution (Xanthine) (Dissolve 1.64mg of Xanthine in 90 mL ddH_2O and while stirring add small amounts of 1N KOH until all of the xanthine has dissolved). Add ddH_2O to a final volume of 100 mL.

e) Xanthine Oxidase Enzyme Solution (XOD): prepare a solution with 5 U/mL XO in ice cold ddH_2O and place on ice.

f) Immediately before use, prepare a solution of 0.05 U/mL XOD in ice cold ddH_2O. This concentration may need to be properly adjusted in order to meet the requirements for the assay as described in the following section.

g) SOD Enzyme Solution. Immediately before use prepare a 10 U/mL solution of SOD in ice cold ddH_2O and place on ice.

Procedure

1. Prepare a Master Solution by pipetting the following reactants at the respective volumes (in mL):

ddH_2O	23
Buffer (a)	25
Buffer (b)	1
Buffer (c)	1
Buffer (d)	50

Mix and adjust the pH to 7.8 at 25°C with 1 M HCl or 1 M KOH if necessary.

2. XOD check

Mix the following in cuvettes (in mL):

	Blank	**XOD**
Master Mix	2.8	2.8
ddH_2O	0.2	0.1
Buffer (f)	-	0.1

Before the addition of the ddH_2O and the Buffer (f), the cuvettes are equilibrated at 25°C and the absorbance is monitored at 550 nm until constant. Subsequently, the ddH_2O and the Buffer (f) are added. The absorbance is monitored at 550 nm for 5 min. The change in absorbance for the uninhibited versus the blank should be 0.025 +/- 0.005 for this reaction. If it is not, adjust the concentration of Buffer (f) and repeat this step until a rate of 0.025 +/- 0.005 is achieved.

3. Pipette (in mL) the following reagents into suitable cuvettes:

	Blank	Uninhibited	Test-1	Test-2	Test-3
Master Mix	2.8	2.8	2.8	2.8	2.8
ddH_2O	0.2	0.1	-	0.01	0.02
Buffer (g)	-	-	0.1	0.09	0.08
Buffer (f)	-	0.1	0.1	0.1	0.1

Buffer (f) is added in the end after an equilibration step of the reactants at 25°C and once the absorbance at 550 nm has been checked to be constant.

The reactants are mixed by inversion and the increase in absorbance is recorded at 550nm for approximately 5 minutes. Make sure to obtain the fastest linear rate over a one minute interval for the uninhibited reaction. Using this time interval, obtain the rates for each Test and Blank.
The ΔA_{550nm} for each inhibited test should fall within the range of 40-60% of the uninhibited rate. Any value outside this range is considered invalid.

4. Calculations

$$\%Inhibition = \frac{(\Delta A_{550nm} / \min Uninhibited - \Delta A_{550nm} / \min Inhibited)(100)}{(\Delta A_{550nm} / \min Uninhibited - \Delta A_{550nm} / \min Blank)}$$

$$Units / mL = \frac{(\%Inhibition)(DF)}{(50\%)(0.1)}$$

DF = Dilution Factor
50% = Inhibition of the rate of cytochrome c reduction per the unit definition
0.1 = Volume (in milliliters) of enzyme used in each test

In a 3.00 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.1 mM EDTA, 0.01 mM cytochrome c, 0.05 mM xanthine, 0.005 unit XOD and 1 unit SOD. The reaction volumes can be adjusted according to the experimental needs maintaining the same final concentrations of the reactants.

c. Pyrogallol-based assay

Pyrogallol can autoxidize in alkaline solutions to produce $\bullet O^{2-}$ anion radicals. The products of the autoxidation i.e. purpurpogallin and superoxide radicals ($\bullet O^{2-}$) can be detected by a spectrophotometer by absorbance at 420 nm. The activity of SOD is reflected by the decrease of absorbance at 420 nm since the action of the enzyme will inhibit the $\bullet O^{2-}$.

Solutions

(a) 50mM Tris-HCl, 1mM EDTA, pH 8,2 in ddH_2O.

(b) 6.67 mM Stock Pyrogallol Solution in ddH_2O; it can be stored on ice for 2 h.

Similarly to previous cases with SOD assays, the preparation of a reference curve with known concentrations of SOD may facilitate the determination of the activity of unknown samples. The final concentration of pyrogallol in the cuvette which shows the best signal to noise ratio is 0.2 mM. The rate of the pyrogallol autoxidation inhibition is spectrophotometrically monitored at 420 nm.

2. Asparaginase Assay

The activity of L-asparaginase enzymes can be assayed by the so-called Nesslerization method. Nessler's reagent is a solution of potassium tetraiodomercurate(II) which forms complex with ammonia molecules (one of the products of the L-asparaginase activity) and subsequently turns into yellow or dark brown at high ammonia concentrations.

Solutions

a) 50 mM Tris Buffer, pH 8.6 at 37°C (Prepare 100 ml in double deionized water using Trizma Base. Adjust to pH 8.6 at 37°C with 1 M HCl.)

b)189 mM L-Asparagine Solution (Prepare 10 ml in double deionized water using L-Asparagine)

c) 6 mM Ammonium Sulfate Standard Solution ($(NH_4)_2SO_4$ Std) (Prepare 100 ml double deionized water using Ammonium Sulfate)

d) 1.5 M Trichloroacetic Acid (TCA) (Prepare 10 ml in double deionized water using Trichloroacetic Acid, 6.1 N Solution)

e) Nessler's Reagent (Ammonia detection reagent).

f) Asparaginase Enzyme Solution (Immediately before use, prepare a solution containing 2.0 - 4.0 units/ml of Asparaginase in cold deionized water)

Procedure

1. Pipette (in mL) the following reagents into suitable tubes:

Reagent	Test	Blank	Std 1	Std 2	Std 3	Blank
a	1	1	1	1	1	1
b	0.1	0.1	-	-	-	-
c	-	-	0.25	0.5	1	-
ddH_2O	0.9	0.9	0.85	0.6	0.1	1.1

Equilibrate to 37°C and then add:

f	0.1	-	-	-	-	-	-	-

Mix by inversion and incubate at 37°C for 30 minutes. Then add:

d	0.1	0.1	0.1	0.1	0.1	0.1
f	-	0.1	-	-	-	-

Mix by inversion and centrifuge for 2 minutes to clarify.

2.

Pipette (in mL) the following reagents into suitable containers:

ddH_2O	4.3	4.3	4.3	4.3	4.3	4.3
Supernatant from step 1	0.2	0.2	0.2	0.2	0.2	0.2
Nessler's reagent	0.5	0.5	0.5	0.5	0.5	0.5

Immediately mix by inversion and after 1 minute record the A_{436nm} for Standards, Tests, and Blanks.

Calculations

Standard curve: A_{436nm} Standard = A_{436nm} Standard - A_{436nm} Standard Blank

Prepare a standard curve by plotting the A_{436nm} of the standard versus ammonia (NH_3) concentration. Note that 1 mole of smmonium sulfate corresponds to 2 moles of Ammonia, therefore a 6 mM ammonium sulfate standard is equivalent to a 12 mM ammonium standard.

Sample Determination: A_{436nm} Test = A_{436nm} Test - A_{436nm} Test Blank

Determine the μmoles of NH_3 liberated using the standard curve.

$$U / mL = \frac{(NH_3 \mu moles)(2.2)}{(0.2)(30)(0.1)}$$

2.20 = Volume of step 1
0.2 = Volume of step 1 used in step 2
30 = Time of assay in minutes
0.1 = Volume (in mL) of enzyme used

Specific activity (U/mg protein):

$$U / mg = \frac{U / mL}{mg / mL}$$

Unit definition: One unit will liberate 1.0 μmole of ammonia from L-asparagine per minute at pH 8.6 at 37°C.

Final assay concentration: In a 2.20 ml reaction mix, the final concentrations are 23 mM Tris-Cl, 8.6 mM L-asparagine and 0.2 - 0.4 units of asparaginase.

The final assay volume can be adjusted according to the experimental needs and the availability of suitable equipment.

3. Lipase Assay

Solutions

a) 0,1M Tris-HCl pH=8,2 (in ddH_2O)

b) 420 µM p-Nitrophenyl laurate / 4-nitrophenyl dodecanoate solution:

- 2,025 mg p-nitrophenyl laurate
- 2,55 mg SDS
- 0,15 g Triton-X
- 15 mL ddH_2O

c) 0,5 mM P-Nitrophenol standard solution

- 0,052 g P-nitrophenol
- Bring to 15 mL with buffer a
- Use 1 mL from above and add 49 mL buffer a for a 0.5 mM stock solution

Procedure

1. For the generation of a Standard curve (range 5-60 uM P-nitrophenol), pipette in mL the following:

Reagent	1	2	3	4	5	6	7	8	9	10
c	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
a	4.95	4.9	4.85	4.8	4.75	4.7	4.65	4.6	4.55	4.5

As Blank, buffer a is used and the absorbance is immediately monitored at 410 nm. The values obtained for each sample are used for the generation of the standard curve.

2. For the determination of lipase activity from a sample, pipette the following in µL into a suitable container:

Reagent	Blank	Test
a	250	250
b	250	250
ddH_2O	100	-
Sample	-	100

The reaction initiates with the addition of the sample into the container. The reaction is continuously monitored spectrophotometrically at 410 nm for 10 minutes.

Calculations

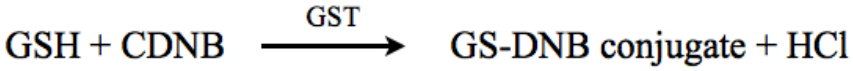
The calculation of U/mL is done using the formula:

$$U / mL = \frac{\Delta A * V_{tot} * 1}{\Delta t * V_{test} * e}$$

V_{tot} = 0.6 mL final volume
V_{test} = volume of sample used
e = slope of the standard curve from step 1

4. Glutathione transferase Assay

The determination of the glutathione S-transferase activity relies on the following 1-step reaction:



GST catalyzes the conjugation of L-glutathione (GSH) to 1-Chloro-2,4-dinitrobenzene (CDNB) through the thiol group of the glutathione. The reaction product, GS-DNB conjugate, absorbs at 340 nm. The rate of increase in the absorption is directly proportional to the GST activity in the sample.

Solutions

a) Sample Buffer: 0.1 M KH_2PO_4 in ddH_2O, pH= 6,5

b) 200 mM reduced GSH in ddH_2O. It is recommended to prepare it fresh; yet, for long term storage, the stock solution can be aliquoted and stored at -20 °C.

c) 100 mM CDNB. CDNB is dissolved in ethanol, while it might be necessary to apply ultrasonics for the complete dissolution of the powder.

d) GST control and sample. The sample should be diluted with Sample Buffer in order to be within the range of the assay.

Procedure

A master mix containing both substrates GSH and CDNB can be prepared, from which amounts either from the GST control or the GST sample can be added for subsequent activity determination. Alternatively, amounts of the substrates are added separately to a final volume which meets the experimental needs. The final concentrations are 2 and 1 mM for GSH and

CDNB respectively. A typical example for assay in 1-mL cuvette is shown in the Table below (amounts in µL):

Reagent	Blank	Test
a	980	970
b	10	10
c	10	10
Sample (Unknown or Control with known GST concentration)	-	10

The cuvette is mixed by inversion several times and the absorbance at 340 nm is continuously monitored for 5 minutes.

Depending on the concentration of the GST in the unknown sample, it should be either diluted with Sample Buffer or use higher amounts from the stock; each time adjusting the Sample Buffer such that the final volume is 1 mL.

This assay can be equally used in 96-well plate format if a plate reader is available.

Calculations

The activity (U/mL) of GST is calculated using the following formula after having determined the rate of the reaction (ΔA_{340})/min which is usually done automatically by the spectrophotometer:

$$U / mL = \frac{\Delta A_{340} / \min * V(mL) * dil}{\varepsilon_{mM} * V_{enz}(mL)}$$

where:

dil: the dilution factor of the original sample

ε_{mM} ($mM^{-1}cm^{-1}$): the extinction coefficient for CDNB conjugate at 340 nm; for test in 1 ml cuvette it equals to 9.6 mM^{-1} (path length - 1 cm).

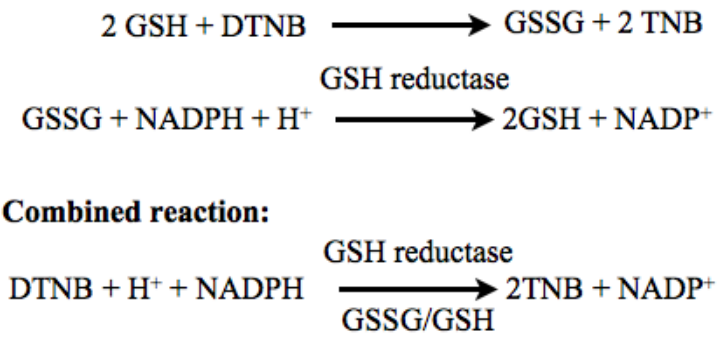
V: the total reaction volume of the assay

V_{enz}: the volume of the enzyme sample used for the assay

5. Glutathione Assay

This assay allows the determination of the total glutathione (GSH + GSSH) level in a biological sample. The measurement of GSH uses a kinetic assay in which catalytic amounts (nmoles) of GSH cause a continuous reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed is recycled by glutathione reductase and NADPH. The GSSG present will also react to give a positive value in this reaction. The yellow product, 5-thio-2-nitrobenzoic acid (TNB) is measured spectrophotometrically at 412 nm. The assay uses a standard curve of reduced glutathione to determine the amount of glutathione in the biological sample.

The full reaction scheme is shown in the Figure below:



Solutions

a) 100 mM KH_2PO_4 , 1 mM EDTA, pH=7 in ddH_2O

b) 1.5 mg/ml DTNB Stock Solution. The solution may be stored in aliquots at –20 °C for at least 3 months.

c) 40 mg/ml NADPH Stock Solution and 0.16 mg/mL working solution. The solution may be stored at –20 °C for at least 6 months.

d) 5% 5-Sulfosalicylic Acid (SSA) Solution. The solution can be kept at 2–8 °C.

e) 10 mM Glutathione (GSH) Standard Stock Solution. The solution may be stored at –20 °C for at least 3 months.

f) Enzyme Solution (GSH Reductase) at 6 Units/mL

Equipment required

- 96 well plate
- plate reader
- multichannel pipette

Procedure

The described procedure below is based on the use of a plate reader which can accommodate 96-well plates. The assay volumes can be adjusted respectively if larger or smaller volumes are required, depending on the availability of equipment in the lab. The final concentration of the reagents should be kept the same with those described here.

Glutathione Standard Solutions: Dilute an aliquot of the GSH Standard Stock Solution (10 mM) 200-fold to 50 µM with the 5% SSA Solution. Prepare the 50 µM solution in the 5% SSA Solution fresh for each standard curve. A GSH solution is significantly more stable in water than in 5% SSA Solution, so the stock solution should always be kept in water at –20 °C.

Prepare the Glutathione Standard Solutions by serial dilution as shown in Table below. Begin with 50 µl of the 50 µM glutathione solution in the first well and then dilute two-fold each time by taking an aliquot of 25 µl from the previous well and adding it to 25 µl of 5% SSA Solution.

Well Number	1	2	3	4	5
GSH Concentration (µM)	50	25	12.5	6.25	3.125
GSH Solution (µL)	50	25 (from well 1)	25 (from well 2)	25 (from well 3)	25 (from well 4)
5% SSA (µL)	None	25	25	25	25
nmoles GSH in a 10 µL sample	0.5	0.25	0.125	0.0625	0.0312

Sample preparation: The biological sample is initially deproteinized with the 5% SSA Solution, centrifuged to remove the precipitated protein, and then analyzed for glutathione. Subsequently, based on the standard curve of reduced GSH, the amount of GSH in the biological sample is

determined. The reaction rate is proportional to the concentration of glutathione up to 2 µM. The plate reader is set at 412 nm with kinetic read at 1 minute intervals for 5 minutes. The reaction scheme for the sample is done as shown in the Table below. Every test should be performed in duplicate.

	Mix and incubate 5 minutes			**Start**
Sample measured	Sample volume	5% SSA	Working mixture	NADPH 0.16 mg/mL)
Reagent Blank	-	10 µL	150 µL	50 µL
Standard curve (different dilutions)	10 µL	-	150 µL	50 µL
Unknown sample	X	10-X	150 µL	50 µL

The final concentration of the components in the reaction mixture is 95 mM potassium phosphate buffer, pH 7.0, 0.95 mM EDTA, 0.038 mg/ml (48 µM) NADPH, 0.031 mg/ml DTNB, 0.115 units/ml glutathione reductase, and 0.24% 5-sulfosalicylic acid.

- The first 2 wells should contain only 10 µl of the 5% SSA Solution as a reagent blank. Add duplicate 10 µl samples of the prepared GSH Standard Solutions into separate wells of the plate. Add varying volumes of the unknown sample in duplicate into separate wells (up to 10 µl sample).

Note: If necessary, adjust the final volume of the unknown sample up to 10 µl with 5% SSA Solution.

- Add 150 µl of the Working Mixture to each well with a multichannel pipette. Mix by pipetting up and down.

- Incubate 5 minutes at room temperature and then add 50 µl of the diluted NADPH Solution with a multichannel pipette. Mix by pipetting up and down.

- Use the plate reader to measure the absorbance in each well. Subtract the reagent blank value from every measurement, unless software is used that performs this automatically.

Calculations

The values of the GSH Standard Solutions are used to determine the standard curve and calculate the ΔA_{412}/min equivalent to 1 nmole of reduced GSH per well.

Calculate the nmoles of GSH in the unknown sample as follows:

$$\frac{\Delta A_{412} / \min(sample) * dil}{\Delta A_{412} / \min(1nmole) * vol}$$

where:

ΔA_{412}/min(sample) = slope generated by sample (after subtracting the values generated by the blank reaction).

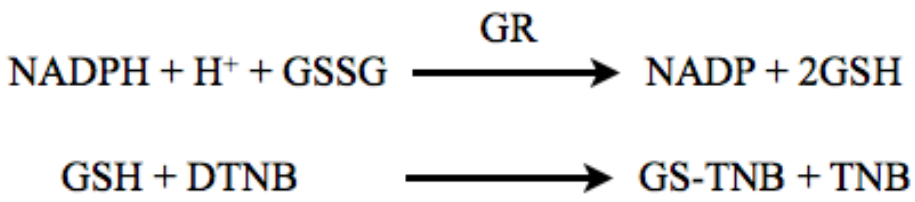
ΔA_{412}/min(1 nmole) = slope calculated from standard curve for 1 nmole of GSH

dil = dilution factor of original sample

vol = volume of sample in the reaction in mL

6. Glutathione Reductase Assay

The glutathione reductase assay allows the determination of the enzymatic activity of the enzyme glutathione reductase (GR) which is present in many tissues. This enzyme catalyzes the reduction of the oxidized glutathione (GSH) in the presence of NADPH and enables the cells to maintain adequate levels of GSH. The principle of this assays is shown in the following Scheme 1, where initially the oxidized GSSG is reduced to GSH by GR and subsequently, the GSH reacts spontaneously with 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB). The activity can be measured either by the decrease in absorbance caused by the oxidation of NADPH at 340 nm (UV assay) or by the increase in absorbance caused by the reduction of DTNB [5,5′-dithiobis(2-nitrobenzoic acid) at 412 nm (Colorimetric assay).



Scheme 1.

Solutions

Prepare the solutions in the volume required depending on the number of assays to be performed and according to the detection method to be used.

a) Solution of glutathione reductase in 100 mM potassium phosphate buffer, 1 mM EDTA, pH 7.5 (Assay Buffer) and 38 mg/mL trehalose. The amount of GR should be adjusted such that, the final activity will be >1 unit per mL in the final buffer. The solution can be aliquoted and stored at -20 °C where it is stable for 6 months.

b) 2 mM β-Nicotinamide adenine dinucleotide phosphate (NADPH) Solution - Reduced (NADPH) is dissolved at final concentration 1.85 mg/ml in Assay Buffer to prepare a working solution of 2 mM. Store at 4 °C. NADPH solution should be prepared fresh every day.

c) 2 mM oxidized glutathione (GSSG) Solution - Dissolve the oxidized glutathione disodium salt at final concentration 1.42 mg/ml in Assay Buffer to prepare a working solution of 2 mM. Store at 25 °C while carrying out the test. The solution can be kept up to 7 days at 2–8 °C for temporary storage.

d) 3 mM 5,5′-Dithiobis (2-nitrobenzoic acid) (DNTB) Solution - Dissolve the DNTB in Assay Buffer to prepare a working solution of 3 mM (1.19 mg/ml). This solution is very unstable; prepare the solution fresh every 4 hours and store at 4 °C.

e) Sample preparation. Dilute the samples to be assayed in 100 mM potassium phosphate buffer, 1 mM EDTA, pH 7.5, 1 mg/mL bovine serum albumin (Dilution Buffer) as needed immediately before assaying. The concentration dependent enzymatic reaction is linear from 0.003–0.03 units per ml of reaction mixture for the colorimetric assay and from 0.003–0.012 units per ml of reaction mixture for the UV assay.

Equipment required

UV/Vis spectrophotometer with thermostatted cuvette holder and a kinetic program. For the UV assay, quartz cuvettes should be used.

Procedure

- Equilibrate the Assay Buffer and 2 mM GSSG Solution at 25 °C for at least 10 minutes before starting the assay.
- Set up a kinetic program in the spectrophotometer with the following recommended parameters:

a) UV assay: Wavelength: 340 nm, Initial delay: 10 seconds, Interval: 10seconds, Number of readings: 11

b) Colorimetric assay: Wavelength: 412 nm, Initial delay 60 seconds, Interval: 10seconds, Number of readings: 11

- Zero the spectrophotometer with a cuvette filled with water.

- Place the following solution volumes in a 1 ml quartz cuvette in the order shown in Table 1.

Solution	UV assay	Colorimetric assay
2 mM GSSG	500 µL	500 µL
Assay Buffer	350-450 µL	50-150 µL
Sample*	0-100 µL	0-100 µL
3 mM DTNB	-	250 µL
2 mM NADPH	50 µL	50 µL
Total volume	1000 µL	1000 µL

* For both (UV or Colorimetric) assays perform a positive control by adding 10 to 20 µL of the Glutathione Reductase Positive Control Solution per control reaction.

-The reaction is started by the addition of the NADPH solution. Mix by inversion, place the cuvette in the spectrophotometer, and initiate the kinetic program.

- As a blank, run the reaction with Assay Buffer instead of enzyme sample solution.

- Calculate the amount of enzyme in the sample.

Calculations

Samples that give a large deviation from linearity should not be taken into consideration. This may happen either with very dilute or with very concentrated samples. The range of enzymatic activity that can be measured varies with the assay used. The concentration dependent enzymatic reaction is linear from 0.003–0.03 units per ml and 0.003–0.012 units per ml of reaction mixture for the colorimetric assay and the UV assay respectively.

The enzymatic activity of the sample can be calculated according to the following formula:

$$Units / mL = \frac{(\Delta A_{sample} - \Delta A_{blank}) * (dilution)}{\varepsilon^{mM} * (volume_{Sample})}$$

where,

ΔA_{sample} and ΔA_{blank}: the rate of the absorbance difference for the sample and the blank respectively

dilution: the dilution factor of the sample

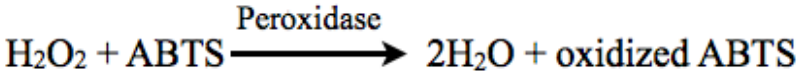
ε: the molar extinction coefficient of NADPH or TNB in mM and

volume: the volume of the sample in mL

For NADPH ε=6.22 $mM^{-1}cm^{-1}$ and TNB ε=14.15 $mM^{-1}cm^{-1}$.

7. Peroxidase Assay

The present assay allows the determination of the peroxidase enzymatic activity based on the following 1-step reaction where the compound 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS) is oxidized in the presence of hydrogen peroxide (H_2O_2) by the peroxidase:



Scheme 1.

The enzymatic activity is monitored spectrophotometrically and continuously at 405 nm which is the absorbance maximum of the oxidized ABTS. The rate of peroxidase activity is proportional to the oxidation of ABTS.

Solutions

a) 100 mM potassium phosphate buffer, pH 5.0 at 25 °C (Prepare 100 mL in deionized water using monobasic potassium phosphate and adjust to pH 5.0 at 25 °C using 1.0 M KOH.)

b) 9.1 mM 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS) substrate solution (Prepare 30 mL in buffer a using ABTS). This solution should always be prepared fresh.

c) 0.3% (w/w) hydrogen peroxide solution (H_2O_2) (Prepare 50 mL in deionized water using H_2O_2 30% (w/w) Solution.This solution should always be prepared fresh.

d) 40 mM potassium phosphate buffer with 0.25% (w/v) Bovine Serum Albumin (BSA) and 0.5% (v/v) Triton X-1002, pH 6.8 at 25 °C (Enzyme Diluent). Prepare 100 mL in deionized water using monobasic potassium phosphate and adjust to pH 6.8 at 25 °C using 1 M K0H.)

e) Peroxidase enzyme solution: Prepare an enzyme stock solution containing 10 mg/mL in cold buffer d. Prepare always fresh a solution containing 0.20 - 0.80 units per mL of peroxidase in cold buffer d.

Procedure

Pipette in mL the following reagents into suitable cuvettes:

Reagent	Test	Blank
Buffer b (ABTS)	2.9	2.9
Buffer d (Enzyme diluent)	-	0.05
Buffer e (Enzyme Solution)	0.05	-

Mix by inversion and equilibrate to 25 °C. Monitor the absorbance at 405 nm until constant, using a suitably thermostatted spectrophotometer and subsequently add:

Reagent	Test	Blank
Buffer c (H_2O_2)	0.1	0.1

Immediately mix by inversion and record the increase in absorbance at 405 nm for approximately 2 minutes. Obtain the ΔA_{405nm}/minute using the maximum linear rate for both the Test and Blank.

Final assay concentrations

In a 3.05 ml reaction mix, the final concentrations are 96 mM potassium phosphate, 8.7 mM ABTS, 0.01% (w/w) H_2O_2, 0.004% (w/v) BSA, 0.008% (v/v) Triton X-100 and 0.01 - 0.04 units peroxidase. The assay volumes can be adjusted according to the experimental needs maintaining the same final concentrations of the components.

Calculations

The units of the enzyme activity are calculated according to the following formula:

$$Units / mL = \frac{(\Delta A_{405nm} Test - \Delta A_{405nmBlank}) * (3.05)(df)}{36.8 * (0.05)}$$

where,

3.05 = Total volume (in milliliters) of assay

df = Dilution factor

36.8 = Millimolar extinction coefficient of oxidized ABTS at 405nm

0.05 = Volume (in milliliter) of enzyme used

Specific activity:

$$Units / mg_{protein} = \frac{units / mL_{enzyme}}{mg_{protein} / mg_{enzyme}}$$

Unit definition: One unit will oxidize 1.0 μmole of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) per minute at pH 5.0 at 25 °C.

8. Glutathione Peroxidase Assay

The present assay describes an indirect method for the determination of glutathione peroxidase (GPx) enzymatic activity. GPx enzymes reduce peroxides to alcohols using glutathione, thereby preventing the formation of free radicals. GPx enzymes will catalyze the reduction of hydrogen peroxide (H_2O_2) and a wide variety of organic peroxides (R-OOH) to the corresponding stable alcohols (R-OH) and water using cellular glutathione as the reducing reagent. The principle of this assay is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced) as shown in the following Scheme 1:

$$\text{R-OOH} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{R-OH} + \text{GSSG} + H_2O$$

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

Scheme 1. GPx is glutathione peroxidase, GR is glutathione reductase, and R-OOH is an organic peroxide.

The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+ is proportional to GPx activity, since GPx is the rate limiting factor of the coupled reaction.

The reaction is performed at 25 °C and pH 8.0, and is started by the addition of the organic peroxide, tert-butyl hydroperoxide (t-Bu-OOH). This substrate is suitable for the assay given the fact that the spontaneous reaction with GSH is low and it is not metabolized by catalase. The reaction with tert-butyl hydroperoxide measures the amount of selenium-containing GPx activity present. If the presence of non-Se enzymes is under consideration and of interest, cumene hydroperoxide can be used as the substrate at a concentration falling within the range of 0.25–1.0 mM. This will measure the total GPx (Se and non-Se enzymes) activity. The difference between the activity observed with cumene hydroperoxide and the tert-butyl hydroperoxide activity is the non-Se glutathione peroxidase activity.

Solutions

a) Glutathione peroxidase assay buffer: 50 mM Tris HCl, 0.5 mM EDTA, pH 8.0.

b) NADPH assay buffer: appropriate amounts of NADPH, reduced glutathione (GSH) and glutathione reductase (GR) are diluted in ddH_2O such that the final concentrations will be 5 mM NADPH, 42 mM GSH and 10 U/mL GR. The solution should be used within 3 hours. It cannot be stored and reused.

c) 30 mM tert-Butyl hydroperoxide buffer.

Equipment required

- UV/Vis spectrophotometer with thermostated cuvette holder and a kinetic program

- Quartz cuvette

Procedure

Initially a glutathione peroxidase standard assay should be performed in order to make sure that the reaction is working. This is done by using purified glutathione peroxidase diluted to a final concentration of 0.25 U/mL in buffer a. From this enzyme preparation use volumes within the range of 20-50 µL to obtain values falling within the linear range of 0.005-0.02 U/mL which in turn, correspond to absorbance decrease of 0.03-0.13 per minute. In cases where the solution of glutathione peroxidase is very pure and/or dilute, the use of IgG in buffer a at final concentration 1 mg/mL is required for stabilization of the enzyme.

Sample preparation: crude biological samples are diluted in buffer a. Several dilutions should be prepared to assure that the reaction rates fall in the linear range.

Pipette in µL the following reagents into 1 mL quartz cuvette as described in Table 1.

Table 1.

	GPx assay buffer	**NADPH assay buffer**	**GPx (0.25 U/mL)**	**Sample**	**30 mM t-Bu-OOH**
Blank	940	50	-	-	10
Positive control	890-920	50	20-50	-	10
Sample	890-930	50	-	10-50	10

1. Pipette the volume of GPx assay buffer indicated in Table 1 into a 1 mL quartz cuvette. The temperature of the assay buffer in the spectrophotometer should be kept at 25 °C by using a thermostated cell holder.
2. Add 50 µL of the NADPH assay reagent and either 10–50 µL of sample or 20–50 µL of enzyme to the cuvette and mix by inversion. The total volume in the cuvette should be 1 mL.
3. Start the reaction by addition of 10 µL of the 30 mM tert-Butyl hydroperoxide solution. Mix by inversion.
4. Follow the decrease in absorbance at 340 nm using a kinetic program. The following program is recommended:

Wavelength: 340 nm
Initial delay: 15 seconds
Interval: 10 seconds
Number of readings: 6

The final concentration of the reagents in the assay mixture is 0.25 mM NADPH, 2.1 mM reduced glutathione, 0.5 U/mL glutathione reductase, and 300 µM t-Bu-OOH.

Calculations

The activity of Glutathione Peroxidase in the sample is calculated based on the ΔA/min at 340 nm which is automatically given by the spectrophotometer, using the following formula:

$$Units / mL = \frac{\Delta A_{340} * (df)}{6.22 * V}$$

where:

$\Delta A340 = A340/min_{(blank)} - A340/min_{(sample)}$
$6.22 = \varepsilon^{mM}$ for NADPH
df = dilution factor of the sample before being added to final reaction
V = sample volume in mL

Unit definition: 1 unit of glutathione peroxidase will cause the formation of 1 µmol of $NADP^+$ from NADPH per minute at pH 8.0 at 25 °C in a coupled reaction in the presence of reduced glutathione, glutathione reductase, and tert-butyl hydroperoxide.

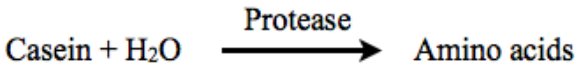
Notes

- Some tissue extracts may contain enzymes that utilize NADPH and skew the results. A blank without t-Bu-OOH can be used as a control for these endogenous activities.
- when using H_2O_2 instead of t-Bu-OOH, the pH of the glutathione peroxidase assay buffer should be adjusted to pH 7 with HCl. At a pH higher than 7 there will be a spontaneous reaction of H_2O_2 with reduced glutathione.
- High concentrations of reducing agents such as DTT and 2-mercaptoethanol (>0.1 mM final concentration in the assay) will underestimate the measured activity by <40% at 0.15 mM and up to 70% at 1 mM concentration in the assay. EDTA at final concentration 5 mM in the assay will underestimate the activity by <50%.

- Nonionic detergents such as TWEEN 20 and Triton X-100 that contain high levels of endogenous peroxides will increase the apparent activity. If these detergents are crucial to the extraction of the proteins of interest, a low peroxide detergent should be used.

9. Protease Assay

This assay allows the determination of protease activity using as substrate casein, which is degraded to amino acids (Scheme 1).



Scheme 1.

This assay is a spectrophotometric stop rate assay which is performed at 37 °C as described in the following sections.

Solutions

a) 50 mM potassium phosphate buffer, pH 7.5 at 37 °C: Dissolve 11.4 mg/mL in purified water using Potassium Phosphate, Dibasic, Trihydrate, Sigma- Aldrich Product Number P5504. Adjust to pH 7.5 at 37oC with 1 N HCl.

b) 0.65% (w/v) casein solution: Dissolve 6.5 mg in 1 mL buffer a and heat gently with stirring to 80-85 °C for approximately 10 minutes until a homogeneous dispersion is achieved. Particular attention should be given not to boil the sample. If necessary, the pH is adjusted to 7.5 at 37 °C with with 0.1 N NaOH or 0.1 N HCl.

c) 110 mM Trichloroacetic Acid solution (TCA): Prepare 1:55 dilution of TCA 6.1 N, approximately 100% (w/v) with purified water.

d) 0.5 M Folin & Ciocalteu's Phenol Reagent (F-C): Prepare a 1:4 dilution of 2 M Folin & Ciocalteu's Phenol Reagent, with purified water.

e) 500 mM sodium carbonate solution (Na_2CO_3): Prepare 53 mg/mL in purified water using anhydrous sodium carbonate.

f) 10 mM sodium acetate buffer with 5 mM calcium acetate, pH 7.5 at 37 °C (Enzyme Diluent). Prepare 1.4 mg/mL trihydrate sodium acetate, and 0.8 mg/mL calcium acetate in purified water. Adjust the pH to 7.5 at 37 °C with 0.1 M acetic acid or 0.1 N NaOH.

g) 1.1 mM L-Tyrosine standard (Std Solution): Prepare 0.2 mg/mL L-Tyrosine in purified water and heat gently until tyrosine dissolves. Similar to casein solution, boiling should be avoided. Cool to room temperature.

h) Protease enzyme solution: Always prepare fresh a solution containing 0.1 – 0.2 U/mL of protease in cold solution f (Enzyme Diluent). For samples where little or no protease detection is expected, prepare sample at 10 mg solid/mL in cold Reagent f (Enzyme Diluent).

Procedure

1. Pipette the following in mL into suitable containers:

Solution	Test 1	Test 2	Test 3	Test 4
b	5	5	5	5

Let the vials equilibrate in a water bath at 37ºC for approximately 5 minutes and then add:

Solution	Test 1	Test 2	Test 3	Test 4
h	1	0.7	0.5	-

Mix and incubate at 37ºC for 10 minutes. Subsequently add:

Solution	Test 1	Test 2	Test 3	Test 4
c	5	5	5	5
h	-	0.3	0.5	1

Mix and incubate at 37ºC for approximately 30 minutes.

Next, filter each solution using a 0.45 µm syringe and use the filtrate in the following step.

Pipette the following reagent in mL into suitable containers: For more consistent results, add solution d immediately following the addition of Na_2CO_3 (Solution e).

Solution	Test 1	Test 2	Test 3	Blank
Test filtrate	2	2	2	-
Blank filtrate	-	-	-	2

e	5	5	5	5
d	1	1	1	1

2. Prepare a standard curve by pipetting the following reagents in mL into suitable containers: For more consistent results, add solution d immediately following the addition of Na_2CO_3 (Solution e).

Solution	Std 1	Std 2	Std 3	Std 4	Std 5	Std Blank
Std solution	0.05	0.1	0.2	0.4	0.5	0
ddH_2O	1.95	1.9	1.8	1.6	1.5	2
e	5	5	5	5	5	5
d	1	1	1	1	1	1

Mix and incubate Blanks, Standards, and Tests at 37ºC for 30 minutes. Remove the containers and allow to cool to room temperature. Next, Filter each Blank, Standard, and Test using a 0.45 µm syringe into suitable cuvette and record the A_{660} nm of each Test, Standard, and Blank solution.

Calculations

- For the Standard curve:

$\Delta A_{660nm(Standard)} = \Delta A_{660nm(Standard)} - \Delta A_{660nm(Standard\ Blank)}$

The values of the absorbance differences ($\Delta A_{660nm(Standard)}$) are plotted vs the µmoles of Tyrosine and the respective Standard curve is obtained.

- For the Sample determination:

$\Delta A_{660nm(Test)} = \Delta A_{660nm(Test)} - \Delta A_{660nm(Test\ Blank)}$ and the enzyme units are calculated according to the following formula:

$$\text{Units/mL} = \frac{\text{µmoles Tyrosine equivalents released} * (11)}{(1) * (10) * (2)}$$

Where:
11 = Total volume of assay in mL
2 = Volume in mL used in colorimetric determination
1 = volume of enzyme used for assay
10 = time in minutes of assay

Specific Activity

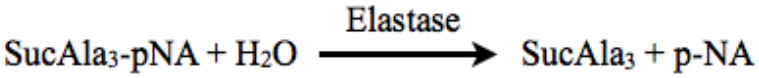
$$U / mg = \frac{U / mL}{mg / mL}$$

Final assay concentrations

In a 6 mL reaction mix, the final concentrations are 42 mM potassium phosphate, 0.54% (w/v) casein, 1.7 mM sodium acetate, 0.8 mM calcium acetate and 0.1 –0.2 units protease. The assay volumes can be adjusted according to the experimental needs maintaining the same final concentrations of the components.

10. Elastase Assay

The present assay allows the continuous spectrophotometric rate determination of the enzymatic activity of the enzyme elastase. It makes use of the substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide ($SucAla_3$-pNA) which is converted by elastase to N-Succinyl-Ala-Ala-Ala ($SucAla_3$)and p-Nitroanilide (p-NA) as shown in the following Scheme 1.



Scheme 1.

Solutions

a) 100 mM Tris-Cl, pH 8.0 at 25 °C: Prepare a 12.1 mg/mL solution purified water and adjust the pH to 8.0 at 25 °C with 1 M HCl.

b) 4.4 $SucAla_3$-pNA substrate solution: Prepare 2 mg/mL solution in Solution a.

c) Elastase enzyme solution: Always prepare fresh a solution containing 0.2–0.5 U/mL of elastase in cold Solution a.

Procedure

Pipette in mL the following reagents into suitable container:

Solution	Test	Blank
a	2.7	2.8
b	0.2	0.2

Mix by inversion, equilibrate to 25 °C and subsequently add:

Solution	Test	Blank
c	0.1	-

Immediately mix by inversion and monitor the increase in A_{410} for approximately 5 minutes. Obtain the ΔA_{410}/minute using the maximum linear rate for both the Test and Blank using a minimum of 4 data points over a one minute time interval.

Calculations

The units of elastase are calculated as follows:

$$Units / mL = \frac{(\Delta A_{410nm} Test - A_{410nm} Blank) * (3) * (df)}{(8.8) * (0.1)}$$

Where:

3 = Total volume of assay in mL
df = Dilution factor
8.8 = mM extinction coefficient of p-Nitroaniline at 410 nm at pH 8
0.1 = Volume (mL) of Enzyme Solution used

Specific Activity

$$Units / mg_{protein} = \frac{units / mL_{enzyme}}{mg_{protein} / mg_{enzyme}}$$

Unit definition

One unit of Elastase will hydrolyze 1 μmole of N-succinyl-L-Ala-Ala-Ala-p-nitroanilide per minute at pH 8 at 25 °C.

Final assay concentrations

In a 3 mL reaction mix, the final concentrations are 96.7 mM Tris-Cl, 0.29 mM N-Succinyl-Ala-Ala-Ala-p-nitroanilide and 0.02–0.05 unit of elastase.

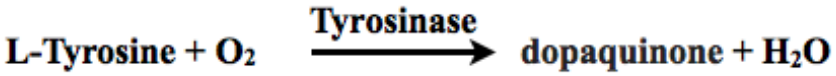
It must be highlighted that the present assay can be used for the determination of the inhibition of elastase enzymatic reaction using different tissue extracts. Such an assay example performed in final volume of 0.6 mL is shown below:

Solution	Blank	Uninhibited	Inhibited
a	0.56	0.54	0.54-X
b	0.04	0.04	0.04
c	-	0.02	0.02
Sample	-	-	X

Initially, the rate of the uninhibited reaction is spectrophotometrically measured at 410 nm as described above and subsequently, it is directly compared with the respective rate of the inhibited reaction. Similar to previous cases of inhibition reactions, the IC_{50} values of the sample of interest can be estimated (One inhibition unit is defined as the amount of sample which causes 50% inhibition of the elastase enzymatic activity).

11. Tyrosinase Assay

The present assay allows the continuous spectrophotometric rate determination of the enzymatic activity of the enzyme tyrosinase. The reaction is shown in the following Scheme 1 where the substrate L-tyrosine is converted to dopaquinone and H_2O.



Scheme 1.

Solutions

a) 50 mM KH_2PO_4 pH 6.5

b) 1 mM L-tyrosine in ddH_2O

c) 500-1000 U/mL tyrosinase in solution a.

Procedure

Pipette in mL the following reagents into suitable container for the preparation of the coctail:

ddH_2O	9
a	10
b	10

Mix and subsequently pipette the following in µL for the performance of the assay:

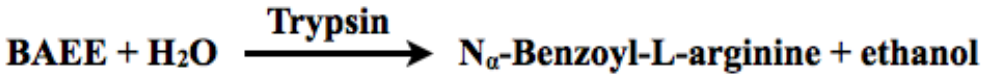
Reagent	Blank	Uninhibited	Inhibited
Coctail	600	576	576-X
c	-	24	24
sample	-	-	X

Rate assay for 5 minutes at 475 nm.

The assay volumes can be adjusted according to the experimental needs maintaining the same final concentrations of the components.

12. Trypsin Assay

This procedure is for products with a specification for trypsin activity using Nα-Benzoyl-L-arginine ethyl ester (BAEE) as a substrate. The procedure is a continuous spectrophotometric rate determination at 253 nm based on the following reaction:



Unit Definition: One BAEE unit of trypsin activity will produce a ΔA_{253} of 0.001 per minute with BAEE as substrate at pH 7.6 at 25 °C in a reaction volume of 1 mL.

Solutions

a) 67 mM NaH_2PO_4 pH=7.6

b) 0.25 mM BAEE

Procedure

Pipette in µL the following reagents into suitable container:

Reagent	Blank	Sample
b	938	938
a	62	-
Sample	-	62

The assay volumes can be adjusted according to the experimental needs maintaining the same final concentrations of the components.

Calculations

$$U / mL = \frac{(\Delta A_{253} / \min Test - \Delta A_{253} / \min Blank) * (df)}{(0.001) * V_{sample}}$$

Where:

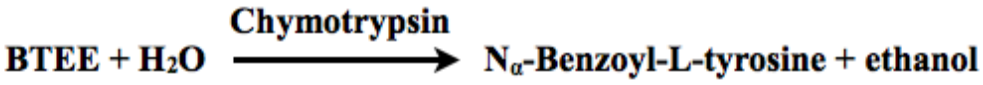
df = dilution factor

0.001 = The change in A_{253}/minute based on unit definition

V_{sample} = volume (ml) of test sample used in assay

13. Chymotrypsin Assay

This procedure is for products with a specification for Chymotrypsin activity. The procedure is a continuous spectrophotometric rate determination at A_{256}, based on the following reaction:



Unit Definition: One unit of chymotrypsin will hydrolyze 1 µmole of BTEE per minute at pH 7.8 at 25 °C.

Solutions

a) 80 mM Tris-Cl, pH=7.8

b) 1.18 mM BTEE dissolved in methanol

c) 2 M $CaCl_2$ in ddH_2O

d) 1 mM HCl

Procedure

Pipette in µL the following reagents into suitable container:

Reagent	Blank	Sample
a	473	473
b	467	467
c	27	27
d	33.5	-
Sample	-	33.5

Rate assay for 5 minutes at 256 nm (UV).

The assay volumes can be adjusted according to the experimental needs maintaining the same final concentrations of the components.

Calculations

$$U / mL = \frac{(\Delta A_{256} / \min Test - \Delta A_{253} / \min Blank) * (df) * V_{reaction}}{(0.964) * V_{sample}}$$

Where:

df = dilution factor

0.001 = The change in A_{253}/minute based on unit definition

V_{sample} = volume (mL) of test sample used in assay

$V_{reaction}$ = total volume (mL) of reaction

0.964= millimolar extinction coefficient of BTEE at 256 nm

14. α-Amylase Assay

Solutions

a) 20 mM NaH_2PO_4, pH=6.9

b) 1 % soluble starch solution. 1 g is mixed with solution a and is boiled for 15 minutes under stirring conditions. Subsequently, it is cooled down and more solution a is added up to 100 mL final volume.

c) Sodium Potassium Tartrate Solution: Dissolve 12 g of Sodium Potassium Tartrate, Tetrahydrate, in previously heated 8 mL of 2 M NaOH, 50°C - 70°C. Heat directly on a heating/stir plate with constant stirring to dissolve. Do not boil.

d) 96 mM 3,5-Dinitrosalicylic Acid Solution: Prepare 20 mL in purified water, 50°C - 70°C, using 3,5-Dinitrosalicylic Acid. Heat directly on a heating/stir plate with constant stirring to dissolve. Do not boil.

e) Color Reagent Solution: To 12 mL of purified water, 50°C - 70°C, slowly add solution c followed by solution d. If not completely dissolved, the reagents should dissolve when mixed. The solution should be stored in an amber bottle at room temperature. The Color Reagent Solution is stable for 6 months.

f) 0.2% (w/v) Maltose Standard: Prepare 10 mL in purified water using monohydrate maltose.

g) Sample

Procedure

Pipette in mL the following reagents into suitable container for the maltose standard:

Reagent	1	2	3	4	5	Blank
f	0.2	0.4	0.6	0.8	1	-
ddH_2O	1.8	1.6	1.4	1.2	1	2
e	1	1	1	1	1	1

Boil for 15 minutes, place on ice for 5 minutes and subsequently add:

ddH_2O	9	9	9	9	9	9

Record absorbance at 540 nm and prepare reference standard curve (mg of maltose vs ABS).

For the Sample analysis, pipette the following in µL:

Reagent	Blank	Sample
b	110	110
g	-	50

Mix and incubate at 20°C for 3 minutes and then add:

e	110	110
g	50	-

Boil at 100°C for 15 minutes, place on ice for 5 minutes and subsequently add in mL:

ddH_2O	1	1

Record the absorbance at 540 nm.

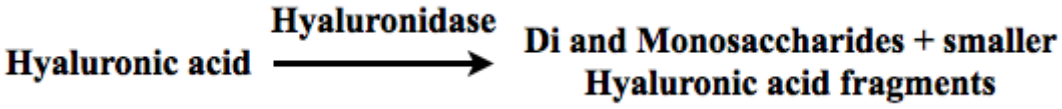
The assay volumes can be adjusted according to the experimental needs maintaining the same final concentrations of the components.

Calculations

Based on the standard curve, the mg of maltose can be calculated

15. Hyaluronidase Assay

This procedure may be used for all Hyaluronidase products. The turbidimetric determination (% Transmittance at 600 nm, Light path = 1 cm) is based on the following reaction:



Unit Definition: One unit of Hyaluronidase activity will cause a change in A_{600} of 0.330 per minute at pH 5.7 at 37 °C in a 2 mL reaction mixture.

Solutions

a) 300 mM Sodium Phosphate, pH 5.35 at 37 °C
b) 0.03% (w/v) Hyaluronic Acid Solution
c) Enzyme Diluent in 20 mM Sodium Phosphate with 77 mM Sodium Chloride and 0.01% (w/v) Bovine Serum Albumin, pH 7.0 at 37 °C.
d) Acidic Albumin Solution: 24 mM Sodium Acetate, 79 mM Acetic Acid with 0.1% (w/v) Bovine Serum Albumin, pH 3.75 at 25 °C.
e) Hyaluronidase Solution: Immediately before use, prepare a 1,000 units/ml enzyme stock solution in cold Enzyme Diluent. Dilute the enzyme stock solution with cold Enzyme Diluent to obtain a working solution of ~6 units/ml.

Procedure

Pipette in mL the following reagents into suitable container:

Reagent	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Blank
e	0.75	0.665	0.585	0.5	0.415	0.335	0
c	0.25	0.335	0.415	0.5	0.585	0.665	1

Mix and equilibrate at 37 °C for 10 minutes and then add:

b	1	1	1	1	1	1	1

- Immediately mix by swirling and incubate at 37 °C for exactly 45 minutes.

- After 45 minutes, transfer 0.5 mL of each Test and Blank into suitable cuvettes containing 2.5 mL of Acidic Albumin Solution and mix immediately by inversion.

- Allow each cuvette to stand for 10 minutes at room temperature.

- Determine the % transmittance at 600 nm.

Zero the spectrophotometer against the Blank and read the % transmittance for the Tests and the Blank. The uncorrected % transmittance for each Test must be between 130–170%.

Calculations

$$U / mL = \frac{(\%T_{Test} - \%T_{Blank}) * (df)}{(14.84) * V_{sample}}$$

where

df: dilution factor of enzyme
14.84: Extinction Coefficient
V_{sample}: Volume of enzyme solution used in reaction

It must be highlighted that the present assay can be used for the determination of the inhibition of hyaluronidase enzymatic reaction using different tissue extracts. In such cases, the IC50 values can be calculated.

16. TBA Assay

Solutions

a) TCA: 20% TCA + 1,5% phosphoric acid + ddH_2O up to 50mL

b) TBA 0,02M (in ddH_2O)

c) TMP 0,2mM (in ddH_2O)

d) TCA:ddH_2O at 1:1 ratio

For the standard curve several dilutions of solution c are prepared in the range of 0.02-0.2 mM. The final volume is adjusted to 1 mL by adding solution d. As Blank, the solution d is used.

Procedure (preparation of the sample)

- Cells are lysed in solution a
- Lysates are centrifuged and the supernatant is collected
- In the supernatants 1 mL of ddH_2O is added
- The final volume is adjusted to 2 mL by adding solution d.

Reaction with TBA

Pipette in µL the following reagents into suitable container:

Reagent	Blank	Sample
b	500	500
d	500	-
Sample	-	500

-

- The samples are mixed and boiled for 35 minutes
- Subsequently, they are placed on ice for 5 minutes
- Finally the absorbance is recorded at 532 nm

Malonaldehyde recovery

Sample preparation

Step A.

- Cells are lysed in a
- Sample is divided into 3 fractions and 30, 60 and 90 µL solution c are added respectively
- 970, 940 and 910 µL ddH_2O are added to the fractions
- Solution d is added in order to reach a final volume of 2 mL
- Samples are centrifuged and the supernatant is collected

Step B.

Several dilutions of solution c are prepared in the range of 0.003-0.009 mM TMP. The final volume is adjusted to 1 mL by adding solution d.

Pipette in µL the following reagents into suitable container:

Reagent	Blank	Sample
b	500	500
d	500	-
Sample	-	500

- The samples are mixed and boiled for 35 minutes
- Subsequently, they are placed on ice for 5 minutes
- Finally the absorbance is recorded at 532 nm

Calculations

The absorbance of the initial samples is used in order to correct the absorbance of the endogenous malonaldehyde.

% recovery=100*ABS_{sp}/ABS_{TMP}

where:

ABS_{sp}=corrected absorbance of endogenous malonaldehyde

ABS_{TMP}= absorbance of the samples in Step B

TBA

$K=\{(1/slope_{standard})*(72.03)*(df)*(10^6)*\%recovery\}/(g\ of\ sample)$

17. Anthocyanins Assay

Solutions

a) 0.025 M KCl, pH=1

b) 0.4 M Sodium acetate, pH=4.5

For each sample two cuvettes are needed. The amount of the sample which can be used is up to 20% i.e. for a final volume of 1 mL, 0.2 mL of sample can be used.

Procedure

Pipette in µL the following reagents into suitable container:

Reagent	1st cuvette	2nd cuvette
a	900	-
b	-	900
Sample	100	100

- The samples are mixed and are incubated for 15 minutes
- Subsequently, the spectrum in the range of 400-700 nm is recorded
- The absorbance at 700 nm (ABS_{700}) and at the wavelength which showed the maximum absorbance (ABS_{max})
- As Blank, ddH_2O is used

Calculations

$A= (A_{max} – A700_{nm})_{pH\ 1} - (A_{max} – A700_{nm})_{pH\ 4,5}$

$mg/L= (A * Mr * df * 1000) /(e * 1)$

where:

Mr=449.2

ε=28000

df: dilution factor

18. Phenolics Assay

Solutions

a) sodium carbonate solution

b) Folin-Ciocalteau (FC) reagent

Procedure

Initially, a reference standard curve using Gallic acid dissolved in methanol is required.

Pipette in μL the following reagents into suitable container:

Reagent	Blank	Sample
ssH2O	800	800-X
FC	50	50
Sample (or for standard)	-	X

The samples are mixed, incubated at RT for 7 minutes and subsequently solution a is added:

a	150	150

The samples are mixed and incubated at RT for 2 h and finally the absorbance is monitored at 765 nm.

19. Enzyme Screening Protocol

Day 1: Preculture preparation

3 strains of bacteria are selected, taken out of the -80C freezer and spread on agar plates inside the laminar flow fumehood. The plates are covered with aluminum foil and left at r.t. over w/end so that colonies grow and the bacteria are returned to the -80 fridge as soon as possible. If there is sufficient growth of isolated colonies on Monday the agar plates are placed in the fridge to prevent further growth until they are used for the preculture, otherwise they are kept at r.t. until sufficient growth is achieved.

Day 2: Marine broth preparation, preculture

37.4g of marine feed is added in three 3L conical flasks and 1L dH2O is added. The flasks are stirred by hand so that the big pieces of marine feed are sufficiently broken to small ones, aluminum foil and indicator tape is placed on the top, and finally they are autocleaved @120C for 20min.

3 falcon tubes are prepared and placed inside the laminar flow fumehood together with the bacteria plates and one of the conical flasks containing the autocleaved marine broth. 10mL marine broth is added to each falcon tube and a sample from an isolated colony of each strain is picked up using the special tool and added to the corresponding falcon tube, taking care to avoid infections. The lid of the falcon tube is relaxed ¼ of a full circle from the closed position and secured with tape, and the falcon tubes are placed in the incubator @20C/180rpm for 24h. The agar plates are kept in the fridge.

Day 3: Culture

The incubated falcon tubes are placed inside the laminar flow fumehood together with the 3L marine broth autocleaved flasks. The content of each falcon tube is poured inside one 3L flask, avoiding contact with the flask wall. The 3L flasks are then placed in the incubator @20C/180rpm over w/end.

Day 4: Centrifugation – filtration of high mw compounds

The cultures are taken off the incubator and poured into 3 1L centrifuge containers which are filled almost completely (the foam can be left out). 1 more container is almost filled with distilled water and the containers are balanced in pairs, closed carefully taking care that the o-rings are in place, and centrifugation follows @6000rpm/4C for 20min. The supernatant is transferred to 1L autocleaved capped bottles and the precipitate (pellets) is transferred using a

spatula to falcon tubes and stored in the fridge. If the pellets contain liquid and the mixture volume is more than 15mL, the falcons are recenrifuged and the supernatant is discarded until a volume of 15mL has been achieved. During this process all fractions waiting to be treated are kept in ice or in the fridge to avoid degradation.

Anti-protease reagent is taken out of the fridge and 10μL are added to each 1L bottle containing the supernatant. The filtration cells are prepared using a 300kD membrane and filled with supernatant solutions as well as 5-10mL of 1M buffer. The filtrations take place in a fridge o/n under 100rpm stirring without the use of pressure, until 300-400mL filtrate is collected. If the filtrate is not enough the next day slight pressure is applied.

Day 5: Cell lysis

The filtration cells are checked to see if the amount of filtrate is enough and if not slight pressure is applied. In the meantime the falcon tubes containing the pellets are slowly defrosted over ice. When the pellet falcon tubes are adequately defrosted they are taken to the cell disruptor together with 3 empty falcon tubes and the anti-protease reagent, all kept in ice. The pressure is set to 1.60 and up to 7mL of mixture is added to the cell disruptor chamber each time. The lysed cells for each strain are poured in an empty falcon tube, the chamber is washed each time with 50mM buffer, the washings are added to the falcon tube so that the final volume is about 50mL for every sample and 10μL anti-protease reagent is added to every falcon tube. The falcon tubes are then centrifuged for 40min @8000rpm/4C and the supernatants are transferred to other empty falcon tubes.

The remaining solutions from the previous filtrations are discarded from inside the filtration cells, the lysed cell supernatant is added, the falcon tubes that contained the lysed cell supernatant are washed with 50mL buffer and the washings are added to the filtration cells The filtration takes place o/n and the filtrate of each strain is added to the previous one of the same strain.

Day 6: Filtration of low mw compounds

The filtration cells are checked to see if the filtration has finished and if not, slight pressure is applied. The filtration cells are then washed and the 300kD filters are removed and stored in 30% EtOH. 10kD filters are installed in the washed cells and the combined filtrates of the previous 300kD filtrations are added. Pressure is applied and the filtration is left o/n taking care of the flow so that the filters are not dried out.

Day 7: Incubation

When almost all the 300kD filtrate in each cell has passed from the 10kD membrane, 100mL 50mM buffer is added to wash the precipitate. Then, the precipitate is collected by successive washings of the filter with 50mM buffer so that the final volume of the washings is 15-20mL. The washings are added in falcon tubes that are kept in ice during the whole process and are stored in the fridge until they are used.

In the meantime, the 96 well 10kD membrane microplates are prepared by addition of 150μL of each polysaccharide aliquot to every well. 1 microplate for the blank and 1 microplate for every tested bacteria strain (usually 3) is prepared. For every microplate, 3 wells are filled with 50mM buffer instead of a polysaccharide aliquot to be used as triplicate control samples.

Then, using the multi channel pipette, 150μL 50mM buffer is added to the blank microplate and 150μL of each 10kD precipitate solution for the microplates that will contain the strains. The plates are then covered with adhesive tape and are left o/n in the incubator which is set at 32C/600rpm. The remaining 10kD precipitate solutions are stored in the fridge to be quantified by Bradford assays.

Day 8: Ferrocyanide reaction – OD measurements

The 10kD membrane microplates are taken out of the incubator, standard 96 well microplates are installed underneath them for collection of the filtrates and the microplate pairs are placed under vacuum for 3h (vacuum should be 15-20mmHg). If the filtration appears to be finished after 3h the pumps are stopped.

The PCR is turned on so that it heats up and a 96 well PCR microplate is prepared by adding 200μL ferrocyanide reagent to each well. Then 40μL of each 10kD filtrate are added to each well using the multi channel pipette and adhesive tape is added on top of the PCR microplate. The PCR microplate is placed firmly in the PCR and a program is set for 95C/15min heating followed by 20C/1min heating. During this time the next PCR microplate can be prepared. After the end of the PCR program the adhesive tape is removed and the PCR microplate is taken out. 200μL of solution from each well are carefully transferred to a standard 96 well microplate using the multi channel pipette and the microplate is ready for OD measurement. In the case of more than 1 strains it is better to measure the OD after the preparation of 2 microplates at most so that the microplates are not left standing for a long time after the ferrocyanide reaction, since the

coloration may change over time. The microplate with the filtrates is kept in the freezer for HPLC analysis.

OD is measured @415nm. The filtrates that show activity are selected and are processed by HPLC (size exclusion column) to verify the presence of oligosaccharides.

20. GC-MS PMAA analysis protocol

Methylation

2 mg polysaccharide are dissolved in 500 mL anhydrous DMSO in a hermetically sealed tube under stirring. After dissolution of the sample, the solution is frozen in an ice bath and 500 mL butyl lithium (BuLi 2.5 M in hexanes, Aldrich) are added. The stirring is continued for 3 h at room temperature. The tube is frozen again and 500 µl iodomethane are added. The stirring is continued overnight at room temperature. The reaction is stopped by addition of 1 mL distilled water and the solution is placed under a nitrogen stream until the lower phase becomes transparent.

Extraction of the methylated polysaccharide

1 mL dichloromethane is added and the tubes are vortexed. The phases are separated by cenrifugation for 1min at 1500 rpm. The aquatic phase is discarded and the organic phase is washed 2 times with 4 mL distilled water. The organic phase is evaporated under a nitrogen stream.

Hydrolysis

500 mL TFA 2 N are added to the solution followed by 20 mL aquatic myo-inositol solution (1 mg/ml) to be used as an internal standard. The tube is closed and placed in a dry bath at 120°C for 2 h. The acid is evaporated under a nitrogen stream.

Reduction

1 mL distilled water and 2 drops NH_4OH are added to the tube as well as a small amount of $NaBD_4$. The solution is vortexed and placed in a dry bath at 80°C for 30 min. After cooling, glacial acetic acid is added dropwise until the gas evolution stops. A mixture of methanol : acetic acid 9 : 1 (v/v, 1 mL) is added and then evaporated under a nitrogen stream. The process is repeated 2 more times.

O-acetylation

200 mL 1-Methyl imidazole and 2 mL acetic anhydride are added to the tube. A yellow precipitate is formed. The tube is sealed hermetically and left for 10 min at room temperature. 5 mL distilled water are added. 2 extractions with 1 mL dichloromethane follow, the organic phases are joined and washed 5 times with 1 mL d'H_2O. The organic phase is placed in a clean tube and evaporated under a nitrogen stream.

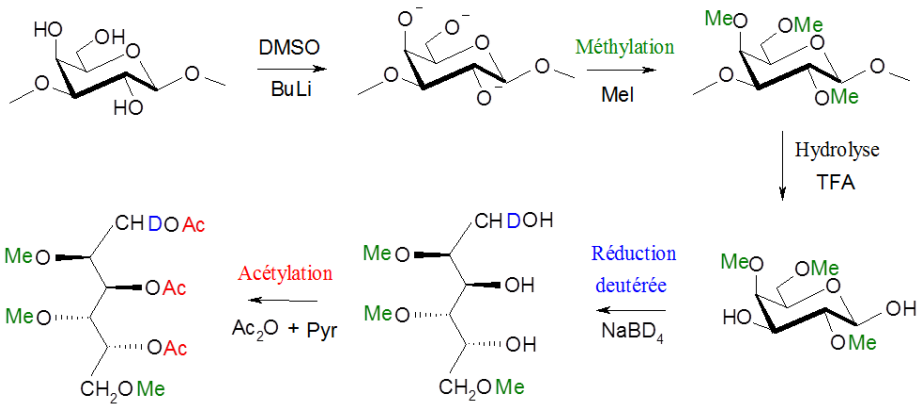


Figure: Formation of the partially methylated alditol acetate of the type 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylhexitol starting from galactose substituted at positions 1 and 3

Screening week 50

21. Screening protocol

Day 1: Preculture preparation (Friday 28/11/14)

3 strains of bacteria are selected, taken out of the -80C freezer and spread on agar plates inside the laminar flow fumehood. The plates are covered with aluminum foil and left at r.t. over w/end so that colonies grow and the bacteria are returned to the -80 fridge as soon as possible. If there is sufficient growth of isolated colonies on Monday the agar plates are placed in the fridge to prevent further growth until they are used for the preculture, otherwise they are kept at r.t. until sufficient growth is achieved.

Day 2: Marine broth preparation, preculture (Thursday 4/12/14)

37.4g of marine feed is added in three 3L conical flasks and 1L dH2O is added. The flasks are stirred by hand so that the big pieces of marine feed are sufficiently broken to small ones, aluminum foil and indicator tape is placed on the top, and finally they are autocleaved @120C for 20min. 3 falcon tubes are prepared and placed inside the laminar flow fumehood together with the bacteria plates and one of the conical flasks containing the autocleaved marine broth. 10mL marine broth is added to each falcon tube and a sample from an isolated colony of each strain is picked up using the special tool and added to the corresponding falcon tube, taking care to avoid infections. The lid of the falcon tube is relaxed ¼ of a full circle from the closed position and secured with tape, and the falcon tubes are placed in the incubator @20C/180rpm for 24h. The agar plates are kept in the fridge.

Day 3: (Friday 5/12/14) Culture

The incubated falcon tubes are placed inside the laminar flow fumehood together with the 3L marine broth autocleaved flasks. The content of each falcon tube is poured inside one 3L flask, avoiding contact with the flask wall. The 3L flasks are then placed in the incubator @20C/180rpm over w/end.

Day 4: (Monday 8/12/14) Centrifugation – filtration of high mw compounds

The cultures are taken off the incubator and poured into 3 1L centrifuge containers which are filled almost completely (the foam can be left out). 1 more container is almost filled with distilled water and the containers are balanced in pairs, closed carefully taking care that the o-rings are in place, and centrifugation follows @6000rpm/4C for 20min. The supernatant is transferred to 1L autocleaved capped bottles and the precipitate (pellets) is transferred using a

spatula to falcon tubes and stored in the fridge. If the pellets contain liquid and the mixture volume is more than 15mL, the falcons are recenrifuged and the supernatant is discarded until a volume of 15mL has been achieved. During this process all fractions waiting to be treated are kept in ice or in the fridge to avoid degradation.

Anti-protease reagent is taken out of the fridge and 10μL are added to each 1L bottle containing the supernatant. The filtration cells are prepared using a 300kD membrane and filled with supernatant solutions as well as 5-10mL of 1M buffer. The filtrations take place in a fridge o/n under 100rpm stirring without the use of pressure, until 300-400mL filtrate is collected. If the filtrate is not enough the next day slight pressure is applied.

Day 5: (Tuesday 9/12/14) Cell lysis

The filtration cells are checked to see if the amount of filtrate is enough and if not slight pressure is applied. In the meantime the falcon tubes containing the pellets are slowly defrosted over ice. When the pellet falcon tubes are adequately defrosted they are taken to the cell disruptor together with 3 empty falcon tubes and the anti-protease reagent, all kept in ice. The pressure is set to 1.60 and up to 7mL of mixture is added to the cell disruptor chamber each time. The lysed cells for each strain are poured in an empty falcon tube, the chamber is washed each time with 50mM buffer, the washings are added to the falcon tube so that the final volume is about 50mL for every sample and 10μL anti-protease reagent is added to every falcon tube. The falcon tubes are then centrifuged for 40min @8000rpm/4C and the supernatants are transferred to other empty falcon tubes.

The remaining solutions from the previous filtrations are discarded from inside the filtration cells, the lysed cell supernatant is added, the falcon tubes that contained the lysed cell supernatant are washed with 50mL buffer and the washings are added to the filtration cells The filtration takes place o/n and the filtrate of each strain is added to the previous one of the same strain.

Day 6: (Wensday 10/12/14) Filtration of low mw compounds

The filtration cells are checked to see if the filtration has finished and if not, slight pressure is applied. The filtration cells are then washed and the 300kD filters are removed and stored in 30% EtOH. 10kD filters are installed in the washed cells and the combined filtrates of the previous 300kD filtrations are added. Pressure is applied and the filtration is left o/n taking care of the flow so that the filters are not dried out.

Day 7: (Thursday 11/12/14) Incubation

When almost all the 300kD filtrate in each cell has passed from the 10kD membrane, 100mL 50mM buffer is added to wash the precipitate. Then, the precipitate is collected by successive washings of the filter with 50mM buffer so that the final volume of the washings is 15-20mL. The washings are added in falcon tubes that are kept in ice during the whole process and are stored in the fridge until they are used.

In the meantime, the 96 well 10kD membrane microplates are prepared by addition of 150μL of each polysaccharide aliquot to every well. 1 microplate for the blank and 1 microplate for every tested bacteria strain (usually 3) is prepared. For every microplate, 3 wells are filled with 50mM buffer instead of a polysaccharide aliquot to be used as triplicate control samples.

Then, using the multi channel pipette, 150μL 50mM buffer is added to the blank microplate and 150μL of each 10kD precipitate solution for the microplates that will contain the strains. The plates are then covered with adhesive tape and are left o/n in the incubator which is set at 32C/600rpm. The remaining 10kD precipitate solutions are stored in the fridge to be quantified by Bradford assays.

Day 8: (Friday 12/12/14) Ferrocyanide reaction – OD measurements

The 10kD membrane microplates are taken out of the incubator, standard 96 well microplates are installed underneath them for collection of the filtrates and the microplate pairs are placed under vacuum for 3h (vacuum should be 15-20mmHg). If the filtration appears to be finished after 3h the pumps are stopped.

The PCR is turned on so that it heats up and a 96 well PCR microplate is prepared by adding 200μL ferrocyanide reagent to each well. Then 40μL of each 10kD filtrate are added to each well using the multi channel pipette and adhesive tape is added on top of the PCR microplate. The PCR microplate is placed firmly in the PCR and a program is set for 95C/15min heating followed by 20C/1min heating. During this time the next PCR microplate can be prepared. After the end of the PCR program the adhesive tape is removed and the PCR microplate is taken out. 200μL of solution from each well are carefully transferred to a standard 96 well microplate using the multi channel pipette and the microplate is ready for OD measurement. In the case of more than 1 strains it is better to measure the OD after the preparation of 2 microplates at most so that the microplates are not left standing for a long time after the ferrocyanide reaction, since the

coloration may change over time. The microplate with the filtrates is kept in the freezer for HPLC analysis.

OD is measured @415nm, the data is exported to a txt file and is inserted manually from the txt file to an excel sheet. The filtrates that show activity are selected and are processed by HPLC (size exclusion column) to verify the presence of oligosaccharides.

22. GC-TMS analysis protocol

Preparation of silylated samples for GC analysis

400µg sample (100µL 4g/L stock) + 40µg myo-inositol (40µL 1g/L stock) are placed in a srew-cap glass tube and the solution is freeze-dried. 500µL 3N MeOH/chlorohydric acid are added and the tubes are heated in a dry bath at 110°C for 4h (capped tightly to avoid evaporation).

After cooling down at r.t., silver carbonate is added for neutralization and 50µL acetic anhydride is added in order to reacetylate potential osamines. The sample is kept o/n in darkness at r.t.

The vials are centrifuged for 15min at 3000rpm. The supernatants are transferred in clean glass tubes and evaporated under nitrogen flow.

100µL pyridine and 100µL sylon (BSTFA:TMCS, 99:1 Supelco) is added and the vials are incubated at r.t. o/n. Evaporation under nitrogen flow follows, 600µL DCM is added and the mixture is filtered (0.22µm nylon syringe filters) into GC vials.

Samples are injected in triplicates using the following methods:

TMS derivatives

120°C 1min

120°C->180°C @ 3°C/min

180°C->200°C @ 2°C/min

200°C for 5min

N-acetyl-osamines

120°C 1min

120°C->150°C @ 2°C/min

150°C->200°C @ 1°C/min

200°C for 5min

23. Bradford protocol

Reagents

1. Dye reagent 5X
2. Stock BSA solutions for c.c.

Stock BSA solutions preparation

High concentration

BSA g/L	0.05	0.1	0.2	0.4	0.75	1
BSA 1g/L µL	50	100	200	400	750	1000
H2O µL	950	900	800	600	250	0

Low concentration

BSA g/L	0.01	0.02	0.04	0.06	0.08	0.1
BSA 0.1g/L µL	100	200	400	600	800	1000
H2O µL	900	800	600	400	200	0

Procedure

1. Remove 5X dye reagent and BSA stock solutions from freezer, allow them to reach r.t.
2. Prepare 4g/L sample solution(s)
3. Prepare 2g/L sample solution(s) by adding to an eppendorf tube 50µL H2O and 50µL from the 4g/L sample solution
4. Prepare in a falcon tube 1X dye reagent from 5X dye reagent by diluting as follows:

3mL 5X + 12mL H2O (1 sample)

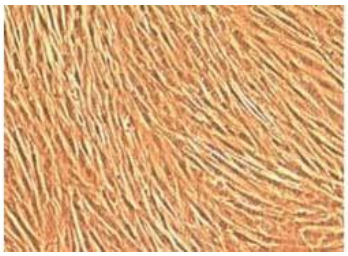
4mL 5X + 16mL H2O (2 samples)

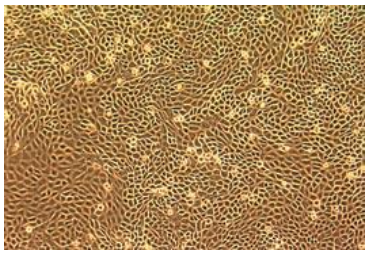
6mL 5X + 24mL H2O (3 samples)

5. Prepare 13 eppendorf tubes (7 for c.c. and 3+3 for sample triplicates) if 1 sample is analyzed, 3+3 more for every extra sample

6. Add 20μL of the 6 BSA stock solutions and 20μL H2O for the blanc to each of the 7 c.c. eppendorf tubes
7. Add 20μL of the sample solutions to the 3+3 sample eppendorf tubes to make triplicates for every sample in both concentrations
8. Add 1mL 1X reagent to all tubes and vortex
9. Incubate for 5-10min at least (not more than 1h at r.t.)
10. Transfer all solutions to cuvettes and measure absorbance @595nm

24. Human Fibroblasts and Keratinocyte Cell Systems





Unpacking and Storage Instructions

1. Check all containers for leakage or breakage.

2. For cryopreserved cells – remove cryovials from the dry ice packaging and immediately place into liquid nitrogen storage. Alternatively, thaw and use the cells immediately. If no dry ice remains, please contact Customer Service.

3. For proliferating cells – Swab down the flask of proliferating cells with 70% ethanol or isopropanol, then place the flask in 37°C, 5% CO2 , humidified incubator and allow to equilibrate for three to four hours. After cells have equilibrated, remove shipping medium from the flask and re- place with fresh medium.

4. BulletKitTM Instructions: Upon arrival, storeBasal Medium at 2°-8°C and SingleQuotsTM at -20°C in a freezer that is not self-defrosting. If thawed upon arrival, growth factors can be stored at 2° -8°C and added to the basal medium within 72 hours of receipt. After SingleQuotsTM are added to basal medium, use within one month. Do not re-freeze.

5. ReagentPack™ Subculture Reagents are sterile-filtered and then stored at -20°C until shipment. Subculture reagents may thaw during transport. They may be refrozen once. If you plan to use within 3 days, store at 2-8°C. Trypsin/EDTA Solution has a limited shelf life or activation at 2-8°C. If, upon arrival, Trypsin/EDTA is thawed, immediately aliquot and refreeze at -20°C. We recommend that the HEPES-BSS and the Trypsin Neutralizing Solution be stored at 2-8°C for no more than one month.

Note: To keep Trypsin/EDTA fresh and active after thawing, you may aliquot it into sterile centrifuge tubes and re-freeze at -20°C

Preparation of Media

For the preparation media, the following steps are performing:

1. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol.
2. Aseptically open each supplement vial and add the entire amount to the basal medium with a pi- pette.
3. Rinse each cryovial with the medium. It may not be possible to recover the entire volume listed for each cryovial. Small losses, even up to 10%, should not affect the cell growth characteristics of the supplemented medium.
4. Transfer the label provided with each kit to the basal medium bottle being supplemented. Use it to record the date and amount of each supplement added.
5. Record the new expiration date on the label based on the shelf life.

Thawing of Cells / Initiation of Culture Process

1. The recommended seeding density for NHDF, HPdLF, AoAF and NHCF is 3,500 cells/cm2 and the recommended seeding density for NHLF and DHLF is 2,500 cells/cm2.
2. To set up cultures calculate the number of ves- sels needed based on the recommended seeding density and the surface area of the vessels being used. Do not seed cells into a well plates directly out of cryopreservation. Add the appropriate amount of medium to the vessels (1ml/5 cm2) and allow the vessels to equilibrate in a 37°C, 5% CO2 , humidified incubator for at least 30 minutes.
3. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure, then retighten. Watch your cryovial closely; when the last sliver of ice melts remove it. Do not submerge it completely. Thawing the cells for longer than 2 minutes results in less than optimal results.
4. Quickly thaw the cryovial in a 37°C water bath being careful not to submerge the entire vial.
5. Resuspend the cells in the cryovial and using a micropipette, dispense cells into the culture ves- sels set up earlier. Gently rock the culture ves- sel to evenly distribute the cells and return to the incubator.
6. Centrifugation should not be performed to re- move cells from cryoprotectant cocktail. This action is more damaging than the effects of DMSO residue in the culture

Subculturing

The following instructions are for a 25 cm2 flask. Adjust all volumes accordingly for other size flasks.

Preparation for subculturing the first flask:

1. Subculture the cells when they are 70 to 80% confluent and contain many mitotic figures throughout the flask
2. For each 25 cm2 of cells to be subcultured:
3. Thaw 2 ml of Trypsin/EDTA and allow to come to room temperature.
4. Allow 7-10 ml of HEPES Buffered Saline Solution (HEPES-BSS) to come to room temperature.
5. Allow 4 ml of Trypsin Neutralizing Solution (TNS) to come to room temperature.
6. Remove growth medium from 4□C storage and allow to start warming to room temperature.
7. Prepare new culture vessels.
8. Subculture one flask at a time. All flasks following the first flask will be subcultured following an optimization of this protocol based on calculated cell count, cell viability, and seeding density.

In a sterile field:

1. Aspirate the medium from one culture vessel.
2. Rinse the cells with 5 ml of room temperature HEPES-BSS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
3. Aspirate the HEPES-BSS from the flask.
4. Cover the cells with 2 ml of Trypsin/EDTA solution.
5. Examine the cell layer microscopically.
6. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. This entire process takes about 2 to 6 minutes, depending on cell type.
7. At this point, rap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and rap again. If cells still do not detach, wait and rap every 30 seconds thereafter.

8. After cells are released, neutralize the trypsin in the flask with 4 ml of room temperature Trypsin Neutralizing Solution. If the majority of cells do not detach within seven minutes, the trypsin is either not warm enough or not active enough to release the cells. Harvest the culture vessel as described above, and either re-trypsinize with fresh, warm Trypsin/EDTA solution or rinse with Trypsin Neutralizing Solution and then add fresh, warm medium to the culture vessel and return to an incubator until fresh trypsinization reagents are available9. Quickly transfer the detached cells to a sterile 15 ml centrifuge tube.

10. Rinse the flask with a final 2 ml of HEPES-BSS to collect residual cells, and add this rinse to the centrifuge tube.

11. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%.

12. Centrifuge the harvested cells at 220 x g for 5 minutes to pellet the cells.

a. Aspirate most of the supernatant, except for 100-200 □l.

b. Flick the cryovial with your finger to loosen the pellet.

13. Dilute the cells in 2-3 ml of growth medium and note the total volume of the diluted cell suspension.

14. Determine cell count and viability using a hemacytometer and Trypan Blue. Make a note of your cell yield for later use.

15. If necessary, dilute the suspension with the HEPES Buffered Saline Solution (HEPES-BSS) to achieve the desired "cells/ml" and re-count the cells.

16. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

17. Determine the total number of flasks to inoculate by using the following equation. The number of flasks needed depends upon cell yield and seeding density. If seeding into well plates at

this time, the recommended density is 10,000 cells/cm2.

$$\text{Total \# of Flasks to innoculate} = \frac{\text{Total \# of viable cells}}{\text{Growth area} \times \text{Rec. Seeding Densit}}$$

18. Use the following equation to calculate the volume of cell suspension to seed into your flasks.

$$\text{Seeding Volume} = \frac{\text{Total volume of diluted cell suspension}}{\text{\# of flasks as determined in step 18}}$$

19. Prepare flasks by labeling each flask with the passage number, strain number, cell type and date.

20. Carefully transfer growth medium to new culture vessels by adding 1 ml growth medium for every 5 cm2 surface area of the flask (1 ml/5 cm2)

21. After mixing the diluted cells with a 5 ml pipet to ensure a uniform suspension, dispense the calculated volume into the prepared subculture flasks.

22. If not using vented caps, loosen caps of flasks. Place the new culture vessels into a 37□C, humidified incubator with 5% CO2

Maintenance

1. Change the growth medium the day after seeding and every other day thereafter. As the cells become more confluent, increase the volume of media as follows: under 25%confluence then feed cells 1 ml per 5 cm2, 25-45% confluence then feed cells 1.5 ml per 5 cm2, over 45% confluence then feed cells 2 ml per 5 cm2.

2. Warm an appropriate amount of medium to 37°C in a sterile container. Remove the medium and replace it with the warmed, fresh medium and return the flask to the incubator.

3. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer and warm only the required volume to a sterile secondary container.

Note: For human primary keratinocytes, we follow the previously mentioned steps, using KBM as a media.

25. RNA Isolation

For RNA isolation we used an RNA kit by Norgen. Hereby, the steps of this procedure are included as well as some quidelines for working with RNA.

Working with RNA

RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

- The RNA area should be located away from microbiological work stations
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water
- Clean all surfaces with commercially available RNase decontamination solutions
- When working with purified RNA samples, ensure that they remain on ice during downstream applications

Procedure

The RNA isolation procedure consists of 4 steps according to the instruction of Norgen.

1. Cell Lysate Preparation

a. Excise the tissue sample from the animal.

b. Determine the amount of tissue by weighing. Please refer to Table 1 for the recommended maximum input amounts of different tissues. For tissues not included in the table, we recommend starting with an input of no more than 10 mg.

c. Transfer the tissue into a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. Grind the tissue thoroughly using a pestle.

Note: The use of liquid nitrogen is recommended. However, if homogenization without flash-freezing is preferred, proceed to Step 1e.

d. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.

e. Add 300 µL of Solution B7 to the tissue sample and continue to grind until the sample has been homogenized.

Note: Maximum homogenization may be achieved by passing the lysate 5-10 times through a 25 gauge needle attached to a syringe.

f. Using a pipette, transfer the lysate into an RNase-free microcentrifuge tube (not provided).

g. Add 600 µL of Buffer R3 to the lysate. Vortex to mix.

h. Add 20 µL of reconstituted Proteinase K to the lysate, and incubate at 55°C for 15 minutes. Vortex the tubes occasionally during incubation.

i. Spin the lysate for 1 minute to pellet any cell debris. Transfer the supernatant to a new RNase-free microcentrifuge tube (not provided).

j. Add 450 µL of 96 - 100 % ethanol (provided by the user) to the lysate. Vortex to mix.

2. Binding RNA to Column

a. Assemble a column with one of the provided collection tubes

b. Apply up to 650 µL of the lysate with the ethanol onto the column and centrifuge at 14,000 x g (~14,000 RPM) for 1 minute.

Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute.

c. Discard the flowthrough. Reassemble the spin column with its collection tube. d. Depending on your lysate volume, repeat Step 2b and 2c as necessary.

Note: If part of the lysate has not passed into the collection tube after Step 2d and the volume is less than 200 µL, continue to Step 2e without additional centrifugation.

e. Apply 400 µL of Solution W1 to the column and centrifuge for 2 minutes.
f. Discard the flowthrough and assemble the spin column with a new collection tube

3. On-Column DNAse Treatment

Optional: If DNase Treatment is not required, proceed directly to Step 4a of Column Wash.

a. Apply 100 µL of Buffer I1 and 15 µL of DNase I to the column and centrifuge at 14,000 x g (~14,000 RPM) for 1 minute.

Note: Ensure that the entire 115 µL of DNase I mix passes through the column. If needed, spin at 14,000 x g (~14,000 RPM) for an additional minute.

b. After the centrifugation in Step 3a, pipette the flowthrough that is present in the collection tube back onto the top of the column.

Note: Ensure Step 3b is performed in order to ensure maximum DNase activity and to obtain maximum yield of RNA, in particular for small RNA species.

c. Incubate at room temperature for 15 minutes.

4. Column Wash

a. Apply 400 µL of Solution W1 to the column containing the DNase I mix and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

b. Discard the flowthrough and reassemble the spin column with its collection tube. c. Repeat steps 4a and 4b to wash column a second time.

d. Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.

5. RNA Elution

a. Place the column into a fresh microcentrifuge tube (not provided). b. Add 50 µL of Solution E1 to the column.

c. Centrifuge for 2 minutes at 200 x g (~2,000 RPM), followed by 1 minute at 14,000 x g (~14,000 RPM) Note the volume eluted from the column. If the entire 50 µL has not been eluted, spin the column at 14,000 x g (~14,000 RPM) for 1 additional minute.

Note: For maximum RNA recovery, it is recommended that a second elution be performed into a separate microcentrifuge tube (Repeat Steps 5b and 5c).

6. Storage of RNA

The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

Troubleshooting Guide

Problem	Possible Cause	Solution and Explanation
	Incomplete lysis of cells or tissue	Ensure that the appropriate amount of **Solution B7** was used for the amount of cells or tissue.
	Column has become clogged	Do not exceed the recommended amounts of starting materials. The amount of starting material may need to be decreased if the column shows clogging below the recommended levels. See also “Clogged Column” below.
	An alternative elution solution was used	It is recommended that the Elution Solution (**Solution E1**) supplied with this kit be used for maximum RNA recovery.
	Ethanol was not added to the lysate	Ensure that the appropriate amount of ethanol is added to the lysate before binding to the column.
	Ethanol was not added to Solution E1	Ensure that 5.6 mL of 96 - 100 % ethanol is added to the supplied Solution E1 prior to use.
	Low RNA content in tissue used	Different tissues have different RNA contents, and thus the expected yield of RNA will vary greatly from these different sources. Please check literature to determine the expected RNA content of your starting material.
	Insufficient solubilization of tissues	Ensure the lysate is diluted with the appropriate amount of RNase-free water, and that the appropriate amount of Proteinase K is added. Also ensure that the Proteinase K treatment is performed at 55°C for the full 15 minutes.The incubation time can be increased up to 30 minutes if required.
	Insufficient solubilization of tissues	Ensure the lysate is diluted with the appropriate amount of RNase-free water, and that the appropriate amount of Proteinase K is added. Also ensure that the Proteinase K treatment is performed at 55°C for the full 15 minutes.The incubation time can be increased up to 30 minutes if required.
	Maximum amount of tissue exceeds kit specifications	Refer to specifications to determine if amount of starting material falls within kit specifications
	High amounts of genomic DNA present in sample	The lysate may be passed through a 25 gauge needle attached to a syringe 5-10 times in order to shear the genomic DNA prior to loading onto the column. Also, ensure that the on-column DNase treatment is performed if high amounts of genomic DNA are found in the sample.

26. cDNA synthesis

For cDNA synthesis we follow the following steps:

1. Add 1ng-5μg of total RNA, 1μl oligo dT (500μg/ml), 1μl dNTPmix (10mM)
2. Heat mixture to 65^{o}C for 5min and quick chill on ice. Collect the content of tube by brief centrifugation. Add to the tube: 4μl first-strand buffer (5x), 2μl DTT (0.1M), 1μl RNASeOUT (40units/μl)
3. Mix content of tube gently and incubate at 42^{o}C for 2min.
4. Add to tube 1μl of Superscript II RT (200units) and mix by pipetting gently up and down.
5. Incubate at 42^{o}C for 50min.
6. Inactivate the reaction by heating the tube at 70^{o}C for 15min
7. The cDNA is ready!!!

27. ATP Determination

For ATP determination we used the Vialight plus Kit by Lonza.The ViaLight™ plus kit is intended for the rapid and safe detection of proliferation and cytotoxicity of mammalian cells and cell lines in culture by determination of their ATP levels. ATP (adenosine triphosphate) can be used to assess the functional integrity of living cells since all cells require ATP to remain alive and carry out their specialized functions. The kit can be used for the direct assessment of cell numbers as each individual cell contains ATP. ATP can be detected by the assay thus making it a substitute for tritiated thymidine uptake and tetrazolium dye reduction in cell proliferation assays. Any form of cell injury results in a rapid decrease in cytoplasmic ATP levels and the ViaLight™ plus kit may therefore be used to replace a wide range of endpoint measurements in cell viability testingHereby, the instruction of use. The ViaLight™ plus kit offers many advantages over conventional methods by avoiding the use of radioisotopes, by giving greater reproducibility and higher sensitivity, and by being very rapid. In addition, the kit has been formulated to be used with a microtitre plate reading luminometer for full automation of the assay. The kit can also be used with microplate beta counters and tube luminometers.

Principles

The kit is based upon the bioluminescent measurement of ATP that is present in all metabolically active cells. The bioluminescent method utilizes an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin according to the following reaction:

Luciferase

$$\text{ATP+ Luciferin + } O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin + AMP + } PP_i + CO_2 + \text{LIGHT}$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer or beta counter. The assay is conducted at ambient temperature (18°C-22°C), the optimal temperature for luciferase enzymes. Bioluminescence is now the most widely used method for the assay of ATP due to its very high sensitivity, wide dynamic range, and ease-of-use.

Outline of the method

The kit contains all the required reagents to perform the assay.

For additional equipment required to perform the assay please see the equipment section. Recommended culture volume:100 µl per well in a 96 well format.

1. Add Cell Lysis Reagent to extract ATP from cells.
2. Wait 10 minutes to allow full extraction.
3. Add ATP monitoring reagent plus (AMR plus) to generate luminescente signal.
4. Wait 2 minutes to allow full signal development.
5. Read luminescence.

Selection of protocol

In order to select the correct protocol for your assay please determine the answers to the following questions:

1) Is the plate size 96 or 384 wells?
2) Are the cell culture plates compatible with luminescence detection.

Reagent reconstitution and storage

NOTE: Please read this section carefully to ensure optimal performance for your assay.

NOTE: This procedure requires at least 15 minutes to
equilibrate.

The ATP monitoring reagent plus (AMR plus) is supplied as a lyophilized pellet. This is reconstituted in assay buffer (supplied) to produce the working solution for use in the assay.

1. Preparation of ATP monitoring reagent plus (AMR plus)

For 96 well plate:

Add assay buffer into the vial containing the lyophilized AMR plus until the vial is approximately 75% full.

Replace the yellow screw cap and mix gently. Pour the reconstituted reagent into the remaining assay buffer.

Repeat the above process to ensure all the lyophilized reagent has been transferred into the assay buffer.

Allow the reagent to equilibrate for 15 minutes at room temperature to ensure complete rehydration.

NOTE: Use reconstituted reagent within 8 hours, or 24 hours if stored at 2°C-8°C. Unused reagent can be aliquoted into polypropylene tubes and stored at -20°C for up to 2 months. Once thawed, reagent must not be refrozen, and reagents should be allowed to reach room temperature without the aid of artificial heat before use.

2. Cell lysis reagent

This is provided ready for use. Store at 2°C-8°C
when not in use.

3. Assay buffer

This is provided ready for use. Store at 2°C-8°C
when not in use.

Equipment

1. Instrumentation

The ViaLight™ plus kit requires the use of a luminometer or beta counter. The parameters of the luminometer/beta counter should be assessed, and the conditions below used to produce the correct programming of the machine. If the luminometer has temperature control this should be set to 22°C, the optimal temperature for luciferase activity

Microplate luminometers
Read time: 1 second (integrated)

Cuvette/tube luminometers
Read time: 1 second (integrated)

Beta counters
Mode: out of coincidence or luminescence
Read time: 1 second (integrated)

2. Additional equipment and consumables a) 10 ml sterile pipettes

b) Either clear bottomed, white walled tissue culture treated plates* for combined culture and measurement, or opaque white microtitre plates suitable for luminescence measurements.

c) Multichannel micropipettes – 50-200 µl (96 well plates).

Steps for ATP determination

1. Bring all reagents up to room temperature before use.
2. Reconstitute the AMR plus in assay buffer.

Leave for 15 minutes at room temperature to ensure complete rehydration.

3. Remove the culture plate from the incubator and allow it to cool to room temperature for at least 5 minutes.
4. Program the luminometer to take a 1 second integrated reading of each appropriate well.
5. Add 50 µl of cell lysis reagent to each well and wait at least 10 minutes.
6. Add 100 µl of AMR plus to each appropriate well and incubate the plate for 2 minutes at room temperature.
7. Place plate in luminometer and initiate the program.

28. Trancriptomic Analysis (Real-time PCR)

PCR or the Polymerase Chain Reaction has become the cornerstone of modern molecular biology the world over. Real-time PCR is an advanced form of the Polymerase Chain Reaction that maximizes the potential of the technique.

Experimental Design

- Primers Design
- SYBR green assay available

Temperature (^{o}C)	Time (min)	
95	10:00	
95	00:15	1-40 cycles
60	01:00	
95	01:00	
60	00:30	Back to 1 cycle
95	01:00	

29. PCR-based methodology for genome size estimation in the microalgae *Tetraselmis chuii*. Isolation of gDNA for whole genome sequencing

1. Primer design for *Tetraselmis chuii* genome size estimation

Genome size estimation prior to perform genome sequencing by NGS is a very important activity in order to determine an appropriate coverage of reading. The protocol here described is based on a real-time quantitative PCR according to Wilhem, Pingoud and Hahn (Real-time PCR-based method for the estimation of genome sizes. Nucleid Acid Research 31 (10), e56, 2003). Three potential (expected to be single copy genes) were selected for primer design considering sequences available at GenBank for *Tetraselmis chuii*: proliferating cell nuclear antigen (Tch_PCNA; AF012212), high affinity phosphate transporter (Tch_Pho; AF520588), and high affinity nitrate transporter (Tch_Nrt2; HM347523). For each target gene two primer pairs were designed: OUTER primers, for the construction of standard curves, and INNER primers, for real-time PCR. All of them were designed using software OLIGO *v*6.89 (Molecular Biology Insights, Inc) according to the general following criteria: i) the most stable 3'-dimer > -3.5 Kcal/mol, ii) the most stable dimer overall > -10 Kcal/mol, iii) no hairpins with a $\Delta G < -1$ and a Tm > 40, iv) primer efficiency (PE) > 480, v) PE of false priming sites < 20% of total PE, vi) Tm between 78-80 ºC, and vii) optimal annealing/extension temperature for PCR of 68 ºC. In this way, the list of designed primer pairs and the expected amplicon size is shown in the following table:

Primer pair name	Expected amplicon size (bp)
Tch_PCNA_Ou	426
Tch_PCNA_In	168
Tch_Pho_Ou	512
Tch_Pho_In	168
Tch_Nrt2_Ou	421
Tch_Nrt2_In	159

2. gDNA isolation for amplification of standard probes

2.1 Two different 1 l flasks were inoculated with the *T. chuii* strain CCFM-03 (Culture Collection from Fitoplancton Marino) and then cultured in f/2 medium (Guillard and Ryther, 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervaceae* (Cleve). Gran. Can. J. Microbiol. 8, 229-239) for a week under the following controlled conditions: T = 22 ºC, light intensity = 150 $\mu Em^{-2}s^{-1}$, 5% CO_2 supplied with continuous air bubbling.

2.2 A volumen of 20 ml (cell density: ~10^7 cell/ml) was taken for each flask and then centrifuged in parallel using 50 ml Falcon tubes at 5000 g for 10 min.

2.3 Supernatants were removed and cell pellets were resuspended in 500 µl of buffer PL1 from the NucleoSpin® Plant II Kit for Genomic DNA isolation from plant (Macherey-Nagel; Ref: 740770.50). Every cell suspension was then added to a 1.5 ml Safe-Lock tube (Eppendorf; Ref: 0030 123.328) containing ~150 µl of 0.2 mm Stainless Steal Beads (Next Advance; Ref: SSB02).

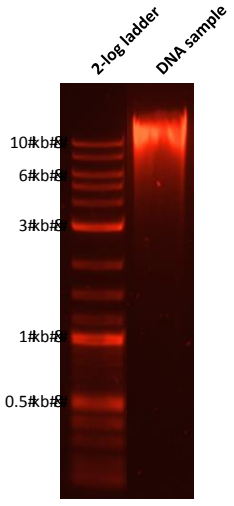
2.4. Cell lysis was performed using the Bullet Blender Cell Disruptor BBX24 (Next Advance) with the following settings: 2 min, speed 5. Samples were then centrifuged at 11000 g for 5 min.

2.5 A volumen of 400 µl of supernatant was recovered from each sample and then 18 µl of RNase A were added (it was included in Macherey-Nagel NucleoSpin® Plant II Kit; stock at 10 µg/µl was prepared following the manufacturer's instructions). Samples were treated for 45 min at 65 ºC using a TS1 ThermoShaker (Biometra).

2.6 From this point on, it was followed the standard protocol described in the user manual from the NucleoSpin® Plant II Kit. However, final elution was performed with 60 µl of PE buffer at 65 ºC, and after 5 min of incubation at 65 ºC, samples were centrifuged at 11000 g for 1 min to elute DNA. They were immediately stored on ice for quantitation using the NanoDrop 2000 Spectrophotometer (Thermo Scientific). Reads were done in duplicates and quality ratios A_{260}/A_{280} and A_{260}/A_{230} checked as expected for good quality DNA samples (between 1.85-1.90 and 1.9-2.1, respectively).

2.7 A standard agarose gel (1% final concentration) in 50 ml TAE 1X buffer (stock: TAE buffer 10X Molecular Biology grade from AppliChem; Ref: A4227.5000) was prepared with 0.5 g of low EEO Agarose (Ecogen; Ref: AG-0120). A sample of the DNAs was loaded in the gel. The 2-Log DNA Ladder (New England Biolabs; Ref: N3200L) was used as molecular weight standard for DNA bands. The non-hazardous 6X dye Bluxyo Safe (gTPbio; Ref: GTP-BM0004) was employed for DNA staining according to manufacturer's instructions.

2.8 Electrophoresis was performed using a Mini-Sub® Cell GT Basic System (Bio-Rad; Ref: 1704380) and a PowerPac™ Basic Power Supply (Bio-Rad; Ref: 1645050) at 80 v during 45 min. DNA bands were visualized using the Gel Doc™ EZ Imager (Bio-Rad; Ref: 1708270) with an UV Sample Tray (Bio-Rad; Ref: 1708271).



A major DNA band higher than 10 kb was observed, with no signal of RNA presence in samples.

3. PCR amplification and purification of standard probes

3.1 The proofreading Velocity DNA polymerase (Bioline; Ref: BIO-21099) was employed for PCR amplification of the three target probes with every pair of OUTER primers (a mix of Forward and Reverse primers at 5 µM final concentration each). A total volumen of 2 x 100 µl was prepared for every PCR, according to the following table (dNTPs mix at 10 mM from Bioline; Ref: BIO-39044):

Buffer 5X	20 µl
Mix primers	8 µl
DMSO	3 µl
Velocity	2 µl
dNTPs 10 mM	10 µl
DNA Tch-1	1 µl
Water	56 µl
TOTAL	100 µl

3.2 PCR reactions were performed using the T100™ Thermal Cycler (Bio-Rad; Ref: 1861096) with the following profile:

(98º 5') + (98º 30'', 68º 30'', 72º 30'') x 35 + (72º 5', 15º 1')

3.3 Bluxyo (20 µl to each PCR) was then added and samples were loaded in an agarose gel as described in 2.7, but at a 0.8% final agarose concentration in a total volume of 150 ml TAE 1X buffer. A Wide Mini-Sub® Cell GT Basic System (Bio-Rad; Ref: 1704469) was employed with a 15 x 10 cm UV-transparent tray and a 20-well comb in which two wells were joined using Scotch tape to create a new double well to allow for loading 120 µl of PCR product plus Bluxyo. This was repeated for every of the six samples (PCR reactions) to be loaded. Electrophoresis conditions were those previously described in 2.8. Then, DNA bands were visualized under a ECX-F15-M transilluminator (Vilber Lourmat) and immediately excised from the agarose gel using Sterile Surgical Blades (Nahita; size 22). Thereafter they were split in small pieces and in each case added to a pre-weighed 1.5 ml eppendorf tube to determine the total agarose weight, which was around 300 mg.

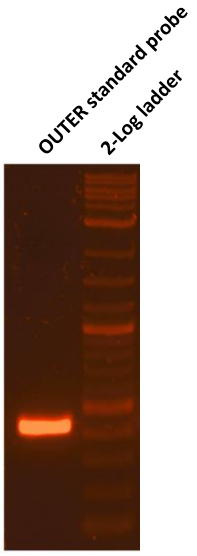
3.4 DNA bands were extracted from agarose gel pieces using the ISOLATE PCR and Gel Kit (Bioline; Ref: BIO-52029) according to the manufacturer's instructions. Final elution was performed in two steps using 25 µl of elution buffer in each one. Thus, a final volume of 50 µl was recovered for every of the six DNA bands, and stored at -20 ºC for further processing

3.5 A second purification step was then performed using the same ISOLATE PCR and Gel Kit following the manufacturer's instructions but with the following modifications: i) to 50 µl a total volume of 500 µl

Binding Buffer A was added before loading in filtration columns, and ii) final elution was performed in two steps, with 15 µl of elution buffer in each, and 5 min incubation at room temperature before centrifugation.

3.6 Samples were quantified using the NanoDrop 2000 Spectrophotometer, with reads in duplicates.

3.7 Both DNA samples corresponding to every OUTER standard probe were mixed and then loaded in an agarose gel as described in 2.7. Electrophoresis conditions were those previously described in 2.8. DNA bands were finally visualized using the Gel Doc™ EZ Imager with an UV Sample Tray (Bio-Rad; Ref: 1708271).



The expected right size of every amplicon was checked using DNA bands in the 2-log ladder as size standards. In the case of Tch_PCNA, a size slightly higher than 500 bp was predicted for the OUTER standard probe, which did not correspond to the expected 426 bp length according to primer pair location (see 1.1).

3.8 The three OUTER standard probes were further sequenced to confirm the right idendity. Double-stranded DNA products were used for direct cycle sequencing using the BigDye® Terminator *v*3.1 kit (Applied Biosystems; Ref: 4337455). All sequencing reactions were performed according to the manufacturer's instructions on a 3130 Genetic Analyzer (Applied Biosystems) using both OUTER and INNER specific primers (that is, a total of four reactions per OUTER standard probe). Alignment of the obtained sequences with those retrieved from GenBank (see 1.1) was performed in MegAlign (DNASTAR). As expected, a 127 bp extra-sequence was found in the Tch_PCNA OUTER standard probe, thus reaching a final size of 553 bp. This putative intron (it contained the standard consensus GT and AG sequences for splicing) had to be considered for estimation of the molecular weight of the OUTER standard probe (see below, section 6) but did no have any effect in further real-time PCR as it was located 5'-upstream of the region were INNER primers were designed.

4. PCR amplification of INNER PCR products using OUTER standard probes as templates

For real-time estimation of *T. chuii* genome size, the protocol here described includes PCR amplification of INNER products using specific primers pairs, as mentioned in 1.1. Thus, it was first checked successfull amplification of those products using in each case the previously isolated OUTER standard probes as DNA templates. Amplification of DNA fragments corresponding to each OUTER standard probe using their specific OUTER primer pair (see 1.1) was also included as a positive control of the system.

4.1 A 1/100 DNA dilution of gDNA was prepared by mixing 99 µl MilliQ water with 1 µl of DNA (see Section 2).

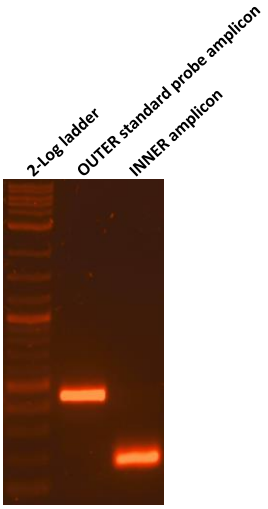
4.2 PCRs were prepared according to the table shown below and run in the the T100™ Thermal Cycler. The iQ™ SYBR® Green Supermix (Bio-Rad; Ref: 1708880) was employed for these tests in standard PCRs as it should be further used in real-time PCRs.

	Volumen
iQ™ SYBR® Green Supermix	12.5 µl
gDNA 1/100	1 µl
Water	10.5 µl
Mix primers	1 µl
TOTAL	25 µl

For every pair of OUTER and INNER primers, a mix of Forward and Reverse primers was prepared at 5 µM final concentration each. PCR profile was at follows:

(95º 5') + (95º 30'', 68º 30'') x 35 + (72º 5', 12º 1')

4.3 DNA samples corresponding to every OUTER standard probe amplicon as well as INNER amplicons were loaded in an agarose gel as described in 2.7. Electrophoresis conditions were those previously described in 2.8. DNA bands were finally visualized using the Gel Doc™ EZ Imager with an UV Sample Tray (Bio-Rad; Ref: 1708271).



The expected right size of every amplicon was checked using DNA bands in the 2-log ladder as size standards.

5. Preparation of gDNA and OUTER standard probe DNA dilutions for real-time PCR

Dilutions of DNA samples were prepared for real-time PCR in order to construct standard curves. Re-quantitation of DNA samples (both gDNA and the three OUTER standard probes) using the NanoDrop 2000 Spectrophotometer was performed again in order to have more accuracy in final calculations.

5.1 The following dilutions were prepared for every OUTER standard probe:

Sample name	Dilution factor
D0	10^{-2}
D1	10^{-4}
D2	10^{-5}
D3	10^{-6}
D4	10^{-7}
D5	10^{-8}
D6	10^{-9}
D7	10^{-10}
D8	10^{-11}

5.2 For gDNA from *T. chuii*, four different dilutions were prepared:

Sample name	Dilution factor
S1	1/100

S2	1/500
S3	1/2500
S4	1/12500

6. Estimation of OUTER standard probes molecular weight

In order to determine the number of molecules of every OUTER standard probe generating a given Ct value in real-time PCR, an estimation of the molecular weight was performed according to location of specific OUTER primers and nucleotide composition as established in Wilhem, Pingoud and Hahn (Real-time PCR-based method for the estimation of genome sizes. Nucleid Acid Research 31 (10), e56, 2003). According to this method, and average weight of 660 g/mol is given to every base pair. Thus, calculation of the mean molar mass (referred to as "M") of every OUTER standard probe was performed as follows:

$$M\ (g/mol) = n^{o}\ bp\ of\ OUTER\ standard\ probe \times 660$$

7. Set-up of real-time PCR plate

7.1 PCR reactions were run in tetraplicates. Dilutions D1 to D8 of every OUTER standard probe (see 5.1) and S1 to S4 of gDNA (see 5.2) were loaded in low profile 96-well Multiplate® PCR Plates™ (Bio-Rad; Ref: MLL9601), and further sealed with Microseal® 'B' adhesive seals (Bio-Rad; Ref: MSB1001). The iQ™ SYBR® Green Supermix (Bio-Rad; Ref: 1708880) was employed in these assays. For every PCR, the following amounts were added:

	Volume
iQ™ SYBR® Green Supermix	5 µl
DNA (standard probe or gDNA)	2 µl
Water	2.4 µl
Mix INNER primers	0.6 µl
TOTAL	10 µl

Mix of every INNER primer pair was prepared as described in 4.2 (5 µM final concentration of each forward and reverse primer).

7.2 The CFX96 Touch Real-Time PCR Detection System (Bio-Rad; Ref: 1855195) was employed with the following profile:

(95º 3.5') + (95º 15'', 68º 30'') x 40 + (95º 1') + (95º 10'', 68º -0.5'')

7.3 Ct values were recorded once run finished for further processing.

8. Calculations for genome size estimation

8.1 As every sample was loaded in tetraplicates, the average of the corresponding four Ct values (avCt) was calculated and further used in calculations for each of D1 to D8 OUTER standard probe dilutions (see 5.1) and *T. chuii* gDNA dilutions (see 5.2).

8.2 For a given OUTER standard probe, the biomass (in g) corresponding to D1 to D8 dilutions was first determined, and thereafter the corresponding number of molecules found in such biomass. The following formulas were applied in each case:

$$\text{Biomass (g)} = [\text{DNA}] \times DF \times 2 \times 10^{-9}$$

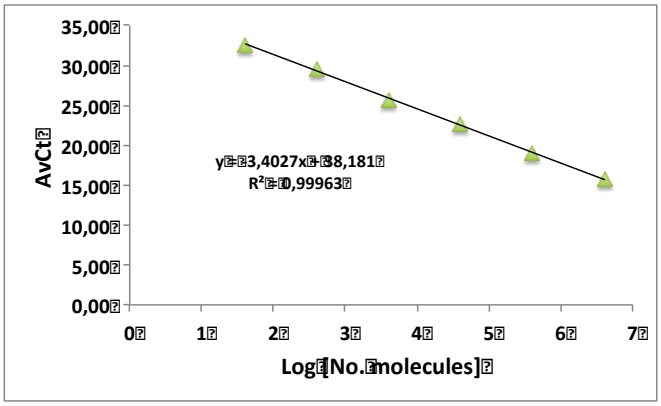
where [DNA] represents the DNA concentration of the OUTER standard probe in ng/µl (see 3.6), and DF is the corresponding Dilution factor of D1 to D8 (see 5.1). The factor "2" in equation was included as 2 µl of DNA were loaded in every real-time PCR reaction (see 7.1), and factor "10^{-9}" was included to transform the final value from ng to g. And then, values for biomass were converted to number of molecules for D1 to D8 as follows:

$$\text{No. molecules} = \text{Biomass (g)} \times N_A/M$$

where "N_A" is Avogadro's number (6.022×10^{23} mol^{-1}) and "M" is the mean molar mass (g/mol) of the OUTER standard probe (see section 6). All values were then transformed to Log [No. molecules].

This process was repeated for every D1 to D8 of the other two OUTER standard probes.

8.3 A standard curve was constructed representing avCt values versus Log [No. molecules]. From the linear regression, the Slope (S) and Intercept (In) were determined. As in some instances D1 and/or D8 were clearly affecting R^2 in the linear regression (the minimum accepted value for R^2 was 0.995), only D2 to D7 were considered as shown in the following example:



Both S and In of the linear regressions were used to calculate the number of copies of the corresponding target marker (Tch_PCNA, Tch_Pho, and Tch_Nrt2) found in a given amount of gDNA.

8.4 For S1 to S4 dilutions of gDNA (see 5.2), the biomass (in ng) loaded in real-time PCR was determined as follows:

$$\text{Biomass (ng)} = [\text{DNA}] \times DF \times 2$$

where [DNA] represents *T.chuii* gDNA concentration in ng/µl (see 2.6), and DF is the corresponding Dilution factor of S1 to S4 (see 5.2). The factor "2" in equation was included as 2 µl of gDNA were loaded in every real-time PCR reaction (see 7.1).

For each of the three target markers, the Log [No. copies] found in every S1 to S4 gDNA dilutions were determined as follows:

$$\text{Log [No. copies]} = (avCt - In)/S$$

where In and S were those determined previously in standard curves. Then, antilog of the obtained values for S1 to S4 were calculated and thus results finally corresponded to No. copies.

8.5 The size of one haploid genome (C), that is, in which only one copy of the target marker is found, was obtained for S1 to S4 from the following formula:

$$C\ (ng) = m \times N^{-1}$$

where "m" is the mass of gDNA template loaded in real-time PCR reactions (in ng) and "N" corresponds to No. copies of the target marker determined by real-time PCR as explained above.

8.6 The haploid genome size, that is, the number of base pairs per haploid genome, is then given by the following formula:

$$\Gamma\ (Mb) = C \times N_A \times M^{-1} \times 10^{-9} \times 10^{-6}$$

where N_A is Avogadro's number (6.022×10^{23} mol^{-1}) and M is the mean molar mass of a base pair (660 g/mol). The factor "10^{-9}" was included to transform the C value from ng to g, and the factor "10^{-6}" was included to obtain the final result in Mb. As for each target marker a Γ value was calculated for gDNA dilutions S1 to S4, the average of these four figures (avΓ) was determined to finally obtain the estimation of haploid genome size for every target marker.

9. Isolation of genomic DNA from *T. chuii* for whole genome sequencing

9.1 Two different 5 l ballons in f/2 medium (Guillard and Ryther, 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervaceae* (Cleve). Gran. Can. J. Microbiol. 8, 229-239) were inoculated from 1 l flask cultures of the *T. chuii* strain CCFM-03 (Culture Collection from Fitoplancton Marino). They were then maintained for a week under the following controlled conditions: T = 22 ºC, light intensity = 150 $\mu Em^{-2}s^{-1}$, 5% CO_2 supplied with continuous air bubbling. Before harvesting, excellent cell mobility as well as absence of bacterial or fungus contamination was checked under microscope.

9.2 Eight 50 ml Falcon tubes (cell density: ~4 x 10^6 cell/ml) were centrifuged at 5000 rpm for 5 min. Supernatants were removed and tubes were filled again with additional 50 ml of culture, and centrifuged at the same conditions previously described. Supernatants were removed and tubes were allowed to dry over a piece of filter paper to remove remaining droplets of media.

9.3 Each pellet was resuspended in 1.8 ml of NIB 1X buffer (MES-KOH 10 mM pH = 5.4, NaCl 10 mM, KCl 10 mM, EDTA 2.5 mM, Sucrose 250 mM, DTT 1 mM, Spermidine 0.5 mM, Spermine 0.1 mM).

Cell suspension was split into two equal volume parts, and each one was added to 1.5 ml Eppendorf Safe-Lock tubes loaded with ~250 µl of 0.2 mm Stainless Steal Beads (Next Advance; Ref: SSB02). Cell lysis was performed using the Bullet Blender Cell Disruptor BBX24 (Next Advance) with the following settings: 3 cycles x [1 min, speed 1], keeping samples on ice for 30 s between cycles. Samples were then centrifuged at 11000 g for 5 min.

9.4 A volume of 800 µl of cell lysate was recovered from each tube, and then 200 µl of a 10% Triton X-100 solution (2% Triton X-100 final concentration) were added and mix softly by pipetting. Samples were incubated on ice for 20 min.

9.5 All tubes were centrifuged at 2000 g for 5 min at 4 ºC. In each case, supernatant was removed and pellet was resuspended in 400 µl of buffer PL1 from the NucleoSpin® Plant II Kit for Genomic DNA isolation from plant (Macherey-Nagel; Ref: 740770.50). Then, 5 µl of RNase A were added (it was included in Macherey-Nagel NucleoSpin® Plant II Kit; stock was prepared at 20 µg/µl following the manufacturer's instructions). Samples were treated for 45 min at 65 ºC using a TS1 ThermoShaker (Biometra), and thereafter centrifuged at 11000 g for 5 min.

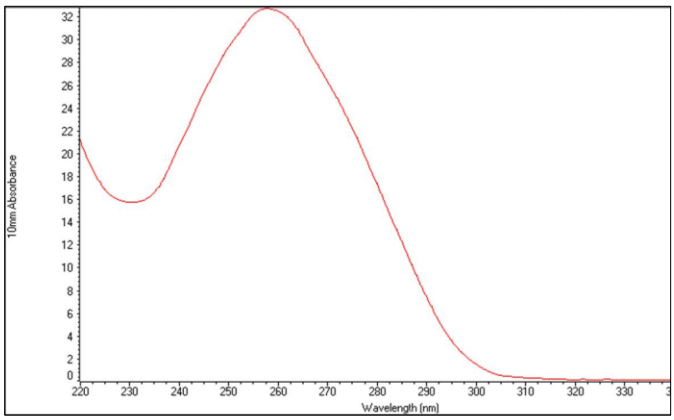
9.6 Supernatants were recovered and further processed according to the standard protocol described in the user manual from the NucleoSpin® Plant II Kit with some modifications: i) a total of eight new 2 ml Eppendorf tubes were prepared, each one containing a mix of 800 µl of supernatant from 9.5 plus 900 µl of buffer PC (final volume: 1.7 ml), ii) a total of eight NucleoSpin® Plant II Columns were employed, loading in each one 1.7 ml from previous step, being necessary three centrifugation steps (with ~650 µl each) at 11000 g for 30 s, iii) two washing steps with buffer PW2 were performed, using 650 µl in each one, followed by a final centrifugation at 11000 g for 2 min to dry the silica membrane completely, and iv) final elution was performed in two steps with a volume of 50 µl buffer PE each. All eluates were joined to reach a final volumen of ~730 µl.

9.7 Final DNA solution was immediately stored on ice for quantitation using the NanoDrop 2000 Spectrophotometer (Thermo Scientific).

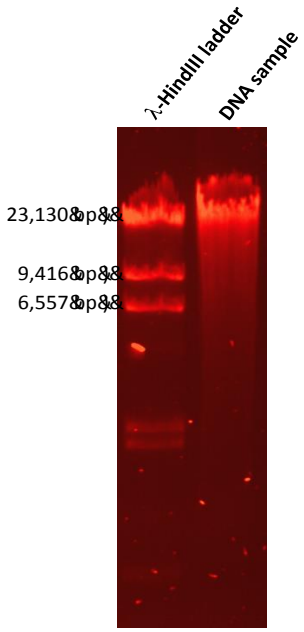
9.8 DNA sample was split into two new 1.5 ml Eppendorf tubes with ~360 µl each, and kept on ice. Then 180 µl of ammonium acetate 7.5 M (final concentration: 2.5 M) at 4 ºC were added to each tube, and softly mixed by pipetting. Thereafter, 1.1 ml absolute ethanol at 4 ºC was added to each tube, and mixed softly by repeated inversion. Tubes were then incubated on ice for 2.5 h to allow for DNA precipitation.

9.9 Samples were centrifuged at 20000 g for 10 min at 4 ºC. Supernatants were carefully removed by pipetting, and DNA pellets were allowed to dry for 15 min at room temperature followed by 3 min at 45 ºC using a TS1 ThermoShaker (Biometra).

9.10 Final resuspension of the two DNA pellets was performed in 50 µl TE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH = 9.0). DNA was quantified again using NanoDrop 2000 Spectrophotometer (Thermo Scientific) in duplicate, and different parameters analyzed as expected for a good DNA quality sample: A_{260}/A_{280} ratio (between 1.85-1.90), A_{260}/A_{230} ratio (between 1.9-2.1), and the absorbance profile (see picture below).



9.11 DNA quality was also checked by agarose gel electrophoresis. A 0.7% standard agarose gel in 50 ml TAE 0.5X buffer (stock: TAE buffer 10X Molecular Biology grade from AppliChem; Ref: A4227.5000) was prepared with 0.35 g of low EEO Agarose (Ecogen; Ref: AG-0120). A DNA sample was loaded in the gel using the non-hazardous 6X dye Bluxyo Safe (gTPbio; Ref: GTP-BM0004) for DNA staining according to manufacturer's instructions. The λDNA HindIII Digest ladder (New England Biolabs; Ref: N3012S) was employed as molecular weight standard for DNA bands. Electrophoresis was performed using a Mini-Sub® Cell GT Basic System (Bio-Rad; Ref: 1704380) and a PowerPac™ Basic Power Supply (Bio-Rad; Ref: 1645050) at 60 v during 90 min. DNA bands (see picture below) were visualized using the Gel Doc™ EZ Imager (Bio-Rad; Ref: 1708270) with an UV Sample Tray (Bio-Rad; Ref: 1708271).



A major band in DNA sample above the highest DNA band of the ladder (23,130 bp) was also indicative of good quality DNA suitable for whole genome se sequencing.