Principles and protocols for the moss *Physcomitrella patens*

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The suitability of mosses as model systems to study plant biology

Mosses (division Bryophyta) are a very successful group of small non vascular green plants widely distributed around the world and able to endure extreme environmental conditions. Their suitability as model systems to study plant cellular and developmental processes was already recognised in the 20s and 30s, and is associated with the following characteristics (for ref., see (1) (2))

(1) Mosses were the first plants to be successfully propagated under axenic conditions and for some species, several generations can be completed in one year on simple mineral media.

(2) Mosses anatomical structures are simple, with relatively few cell types and organs composed of single cell sheets. However, despite this apparent simplicity, mosses display sophisticated morphological structures associated with complex morphogenetic processes.

(3) The haploid gametophyte dominates in the life cycle of Bryophytes, which facilitates mutagenesis and genetic analysis. Yet, sexually fertile diploid gametophyte can be generated by apospory, a process by which sporophytic tissue is induced to regenerate a diploid protonema, and haploidisation of a diploid gametophyte can be obtained in some species following differentiation of spore capsules from unfertilised gametophytic tissue (apogamy).

(4) The gametophyte development can be subdivided in two phases: the protonema stage, which is characterised by a branched network of filamentous cells displaying unidimensional apical growth, and the gametophore stage, which displays three-dimensional caulinary growth processes analogous to that of higher plants. In both phases, physiological and developmental processes can be monitored *in vivo* at the single cell level.

(5) Mosses development is regulated by similar growth substances (such as auxins, cytokinins, abscisic acid, gibberelins) and environmental factors (such as light and gravity) to those regulating higher plants morphogenesis.

(6) Mosses display an incredible regeneration capacity and in some species any part of the plant can regenerate a protonema under appropriate growth conditions. This regeneration is associated with direct differentiation of chloronemal cells from differentiated tissues, and usually does not involve callus formation and dedifferentiation. One can therefore consider that in these species, every single moss cell is developmentally totipotent.

(7) Genetic transformation studies of the *Physcomitrella patens* have revealed that integration of foreign DNA sequences in the genome occurs preferentially at targeted location by homologous recombination (3). Thus, the powerful genetic tools developed in the yeast *Saccharomyces cerevisiae* (4) can be directly applied to study plant biology in the moss *Physcomitrella patens* (5).

These features fulfil some of the major experimental requirements for the establishment of a model system, and no other plants reach such an ideal situation.

Protocols

Plant cultivation

Growth conditions

The standard conditions for the culture of *P. patens* in the lab are as follows. Cultures are grown in the culture room at 26 ± 2 ºC in discontinuous white light (16 hours / day). Light is provided by fluorescent tubes Sylvania GRO-LUX WS at quantum irradiance of 50 to 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and with a red/far-red ratio of 1.5. Protonemal cultures are made in 9 cm Petri dishes poured with solid culture medium and overlaid with 8cm diameter cellophane disks (A.A. Packaging Limited, Liverpool Road, Walmer Bridge, Preston Lancashire PR4 5HY). The cellophane disk is not necessary but facilitate subsequent observations and collection of material.

Sporogenesis is performed in Magenta boxes, or in culture glass tubes on minimal medium (PP-NO3 without glucose). Cultures with well differentiated gametophores are irrigated with sterile water and transferred at 17ºC in illuminated temperature-controlled growth chambers with a short-day photoperiod of 8 hours / day for three weeks to induce gametogenesis. Sporophyte development is further completed under standard conditions (short-day) and their maturation is followed visually.

Strain conservation

Strains are conserved as fragmented protonemal suspension in sterile water in the fridge. These suspensions remain viable for several years. For short term storage, collect a 6 day old protonemal culture in sterile water (1 plate in 5-10 mL) and fragment it with an Ultraturax (Polytron, 30 sec. at position 4). Inoculate plates with freshly fragmented suspension. For long term storage, filter the suspension after fragmentation on a 100-500 µm stainless steel filter. Collect the fragments of protonema that are retain by the filter with a forceps and resuspend them in sterile water.

Spores provide another very convenient mean for long-term storage of moss strains. Collect one single spore capsule (resulting from a single fertilisation event) in 1 mL sterile water in an Eppendorf and store in the fridge. Spore germination has been obtained from 10 year-old spore suspension.

Strains can also be stored for several months in the fridge as colonies on a Petri dish sealed with parafilm. This provides a convenient way for medium term storage of strains without fragmentation (ie directly from the culture room). In this case, one should not fragment the colony prior to inoculation, but simply take a piece of the colony and directly inoculate a PP NH4 plate.

Culture media

Culture media are derived from the recipe described in (6)

PP NO_3

This is the minimal medium; it is used for phenotypical analysis and for the production of spores.

Macro elements:

$CaNO3 \cdot 4H_2O$	0.8 g/l
$MgSO_4 \cdot 7H_2O$	0.25 g/l
$FeSO_4 \cdot 7H_2O$	0.0125 g/l

These elements are weighted and directly added to the medium

Micro elements

$CuSO_4 \cdot 5H_2O$	0.055 mg/l
$ZnSO_4 \cdot 7H_2O$	0.055 mg/l
H_3BO_3	0.614 mg/l
$MnCl_2 \cdot 4H_2O$	0.389 mg/l
$CoCl_2 \cdot 6H_2O$	0.055 mg/l
KI	0.028 mg/l
$Na_2MoO_4 \cdot 2H_2O$	0.025 mg/l

Prepare a 1000 x stock solution, autoclave, store in the fridge and add 1 mL / L medium

Phosphate buffer

Dissolve 25 g KH_2PO_4 in 100 mL water and titrate to pH 7 with 4M KOH to make a 1000x stock. Autoclave and add 1 ml per litre of medium.

PP NH_4

This medium is used for the production of chloronemata enriched protonema used to isolate protoplasts and for rapid large scale amplification. The presence of ammonium tartrate in the medium increases the yield of secondary chloronemata, but does not allow the completion of the life cycle and must be absent for the production of spores.

Add to the minimal medium

NH_4 tartrate	500 mg/l
Glucose	5 g/l

Agar

Add 7 g/l Agar agar (Merck 1614) and sterilise by autoclaving (20 min, 120ºC). A precipitate forms during autoclaving (probably calcium phosphate). Poor the plates after sterilisation and avoid keeping molten medium since the precipitate will be more abundant. Solid plates can be stored for at least one month at room temperature.

Supplements

Supplements for auxotrophs

Thiamine HCl	500 µg/l
Para-aminobenzoic acid	250 µg/l
Nicotinic acid	1 mg/l

Supplements are added to the culture medium from a 1000X stock solution prior to sterilisation

Antibiotic supplement

For neomycin resistance, supplement medium with 50 mg/l G-418.

For hygromycin resistance, supplement with 25 mg/l Hygromycin B.

For sulfadiazine resistance, supplement the medium with 150 mg/l sulfadiazine.

Antibiotic are added to the medium from a 1000x stock solution after autoclaving.

Glucose

5g/L glucose is routinely added to the media to facilitate early detection of putative contamination. But it MUST be absent from sporogenesis cultures. Protonema and gametophores will turn brown and eventually die much earlier on glucose containing medium. It must therefore be omitted for long term cultures, for the production of spores and for phenotypic analysis.

Protoplasts media

Protoplast solid culture medium.

Supplement PP NH_4 solid culture medium with 66 g/l mannitol and check osmolarity (around 480 mOsmol). Sterilise by autoclaving.

Protoplast liquid culture medium.

Supplement PP NH_4 culture medium without agar with 66 g/l mannitol and check osmolarity (around 480 mOsmol). Sterilise by autoclaving. A precipitate forms during autoclaving (probably calcium phosphate). Sediment the precipitate and use the clear supernatant.

Protoplast top layer

Mannitol 8.5 % or protoplasts liquid culture medium with 1.4 % agar

Culture method

Amplification of protonemal tissue for protoplast isolation, biochemical and molecular analysis is performed on PP NH_4 medium. Inoculate 2 ml of a freshly fragmented protonema per 9 cm Petri dishes poured with solid culture medium and overlaid with cellophane disks (W.E. Cannings, Bristol, UK). Petri dishes must not be sealed with parafilm since it considerably reduces growth. Collect material after a week since it starts to turn brown after. The yield is 1 to 1.5 g/Petri dish.

Amplification on PP NO_3 medium is also possible. The yield will be lower, the culture will contain less chloronemata, more caulonemata and more buds, and the culture can be kept in the culture room for up to 5 weeks if the dish is sealed with parafilm to avoid desiccation of the medium.

Phenotypic characterisation is usually performed on minimal medium in the absence of glucose. Density of inoculation, type and volume of culture vessel must be adapted to the situation. Supplementation with growth factors should be tested on both media. 6-benzyladenine and isopentenyladenine are the most commonly used cytokinins, β-indolyl-3-acetic acid or 1-naphtalene acetic acid are used as the auxins.

Protoplast isolation and regeneration

(adapted from (7))

Collect protonema from 5 cultures grown for 5- 6 days on PP NH_4 and transfer to a 9 cm Petri dish containing 10 ml 0.48M mannitol.

Add 10 ml of a sterile solution of 2% (w/v) non purified Driselase (Fluka 44585, Sigma D-9515) and incubate at room temperature for 30 min. with occasional gentle mixing. (Driselase is dissolved in 0.48M mannitol, centrifuged at 10000 rpm for 10 min. to remove debris, buffered to pH 5.6 and sterilised by passage through a 0.20 µm filter).

Filter the preparation through a sterile 100 µm stainless steel sieve and leave for an additional 15 min. to complete digestion.

Filter through a sterile 50 µm stainless steel sieve and transfer to sterile 10 ml glass tubes. Harvest the protoplasts by low speed centrifugation (600 rpm for 5 min.) and gently resuspend the pellet in mannitol 0.48M.

Repeat centrifugation and resuspend the cells in mannitol 0.48M. Take one aliquot of the suspension (eventually dilute ten times in mannitol 0.48M) and counts protoplasts with an hematocytometer. The yield is usually around 10^6 viable protoplasts per initial culture plate. Viable protoplasts are identified by their intact shape, but only ca 50% of these will regenerate. Vital staining at his stage is not required.

Repeat centrifugation and resuspend in mannitol 0.48M at the desired concentration. Mix one volume of protoplast with one volume of molten protoplast top layer and dispense 2 ml aliquot per 9 cm Petri dishes containing protoplasts solid culture medium overlaid with a cellophane disk. Ideal concentration for good regeneration is between 10 000-30 000 protoplasts per Petri dish. Regeneration will be

seriously delayed if there is less than 5000 protoplasts / Petri. Leave the protoplast in darkness one night after isolation and then regenerate in light in the culture room.

Selfing, crossing and segregation analysis

To obtain self progeny, inoculate a piece of a 1 week old protonema onto minimal medium and grow under standard conditions until ca 25 gametophores are well differentiated. Cultures are then irrigated with sterile water and transferred to 17ºC for 3 weeks to induce gametogenesis. The cultures are brought back to 26ºC and the maturation of sporophytes is followed visually. Mature spore capsules (orange brown) are harvested individually prior to dehiscence, crushed in 1 ml sterile water in an Eppendorf to resuspend the spores and kept 1-3 weeks at 4ºC to ensure high spore germination rates. Each capsule contains ca 4000 spores.

Strain *nicB5ylo6* present the advantage of self-sterility associated with auxotrophy for nicotinic acid and with a convenient phenotypic yellow marker (*ylo)* allowing easy identification of this strain in crossing experiments (8). A protonemal inoculum of a transgenic strain and of strain *nicB5ylo6* are transferred side by side on PP-NO_3 medium in glass culture tubes and regenerated at 26ºC until some 25 gametophores are completely differentiated (colony diameter, ca 2 cm). Cultures are then irrigated with sterile water and transferred to 17ºC for 3 weeks to induce gametogenesis. The cultures are brought back to 26ºC and the maturation of sporophytes is followed visually. Mature spore capsules (orange brown) are harvested individually prior to dehiscence, crushed in 1 ml sterile water in an Eppendorf to resuspend the spores and kept 3 weeks at 4ºC to ensure high spore germination rates. Spore capsules collected on the wt or the transgenic colonies are generated by self-fertilisation and the ones collected on the *nicB5ylo6* colony by cross-fertilisation.

To inoculate spores, take 50 µl of the suspension, mix with 2 ml of 0.7% agar in water (molten) and plate it on a 9 cm Petri dish on a cellophane disc. Spore germination occurs within 4 to 5 days and well defined single spore derived colonies can be isolated within 2 weeks. Segregation of the yellow marker can usually be scored within 3 to 4 weeks. Transfer a piece of a well defined single spore derived colony germinated under non selective conditions to antibiotic supplemented medium to score antibiotic resistance segregation.

Transformation of the moss <u>Physcomitrella patens</u>

Some notes will follow the procedure to emphasise the main factors influencing the efficiency of transformation.

Protocol

-To obtain good protoplasts batch, isolate them from a culture inoculated from a freshly fragmented culture.

-Isolate protoplasts from 5-6 days old protonemal culture incubated with 1% Driselase for 30 minutes and then filtered (100 µm sieve).

- Continue digestion for a further 15 minutes and then filter (50 µm sieve).

- Transfer the suspension to sterile glass tubes and sediment the protoplasts by low speed centrifugation for 5 minutes at 600 g.

- Gently resuspend the pellet in 10 ml mannitol 8.5% and repeat centrifugation.

- Resuspend pellet in mannitol and count. Leave the protoplasts in mannitol before initiating transformation (1).

- Recentrifuge and then resuspend the protoplasts at a concentration of 1.2 x 10^6 / ml in MMM solution (2, 3).

- Dispense 10-15 µg of DNA into 14 ml Falcon tubes (in maximum 30 µl, the final concentration should be between 30-50 µg/ml, see 4).

- Add 300 µl of protoplast suspension and mix gently.

- Add 300 µl of PEG solution and mix gently (5).

- Heat shock 5 minutes at 45^o C .

- Bring back to room temperature and leave for an additional 10 minutes with occasional gentle mixing (6).

- Progressively dilute the sample with of moss liquid medium (5 x 300 µl and then 5 x 1 ml added sequentially every minute) (7).

- Keep the transformed protoplasts overnight in darkness

- The nest day, protoplasts may be further cultured in liquid medium for transient gene expression assay or embedded in protoplast top layer and plated on protoplast solid medium for further selection. For the selection of transformants, each transformation sample should be plated on 3-4 Petri dishes.

Notes

(1) Maintaining the protoplasts in MMM solution reduces cell viability.

(2) The MMM solution is required to achieved high transformation efficiency.

(3) Protoplast concentrations of 1 to 1.5 x 10^6 / ml are optimal for transformation efficiency and cell survival.

(4) A large precipitate containing DNA + cellular material will result upon addition of PEG if the DNA concentration is high (< 70 µg / ml). This has been observed for PEG purified plasmid DNA, but may not be true for Qiagen column purified DNA. DNA concentrations lower than 20 µg / ml will reduce the efficiency of transformation.

(5) Mix immediately to avoid the separation of PEG and protoplasts.

(6) Longer incubation in PEG decreases cell survival and specific activity in transient assay.

(7) Do not use the MMM solution since it will decrease cell survival. The same is true if you wash and resuspend the cells before starting cultivation. Moreover, it seems that the cells which have been permeabilised will be preferentially killed by the washing step (as monitored by a decrease in specific activity in transient essay).

Solutions.

Mannitol

Mannitol: 0.48 M (8.5%)

MMM

Mannitol: 0.48 M (8.5%)

Magnesium chloride: 15mM

MES: 0.1%, pH 5.6 with KOH

PEG

Mannitol 0.38 M (7 %)

Calcium nitrate 0.1M

PEG 4000 (Serva) 35-40 % (w.v)

pH 8.0 with 10 mM Tris

Dissolve 4 g of PEG in a final volume of 10 ml of mannitol 0.38M, calcium nitrate 0.1 M. Heat the solution at 45^{o} C to assist PEG dissolution. Buffer the solution with 10 mM Tris pH 7.2 and sterilise it by autoclaving or by filtration through a 0.20 µm filter. The PEG solution should be kept at –20° C. Storage at room temperature will result in PEG hydrolysis which induces a strong drop of pH and a low survival of protoplast. PEG 6000 can be used instead of PEG 4000, but the specific activity will be reduced. Different batches of PEG may have different properties so this should be checked.

General remarks

The mannitol concentration in the PEG solution is an important factor and should be checked for different species.

Heat shock is necessary although some DNA uptake without heat shock has been occasionnaly observed. A specific activity / heat shock dose response has been obtained with increasing time of heat shock.

A specific activity / DNA concentration dose response has been obtained with increasing concentration of plasmid DNA, providing the final concentration is maintained constant with sheared Salmon sperm DNA. Saturation occurs with 20 µg / ml plasmid DNA.

High transient expression is readily measurable after 24 hours of culture, reaching a maximum after ca 40 hours. A decrease in specific activity is measurable after ca 4 days. Using a GFP reporter, 5 to 25 % of the protoplasts are labelled 30 hours after transformation.

Selection for stable transformants should be undertaken using high antibiotic concentrations (ca 10 x LD_{50}). Under these conditions, the growth rate of stable clones is similar to the untransformed wild type plated on non-selective medium, whereas the growth rate of unstable clones is seriously reduced.

Selection conditions, non-selective to selective growth test and rescue experiments

Selection is initiated 6 days after transformation by transferring the top layer to 9 cm Petri dish containing PP NH_4 solid medium supplemented with 50 µg/ml G-418 or 25 µg/ml Hygromycin B, corresponding to ca. 10 times LD_{50}. . After transformation and one week regeneration on mannitol plates, transfer the cellophane for 10 days on selective plates. Then transfer the cellophanes to non-selective plates for 10 days and then return to selective plates for at least 10 days. All unstable clones will display mosaicism for the resistance with dead and resistant sectors.

The non-selective to selective growth test is performed as follows: resistant colonies are fragmented for 30 seconds with a Polytron (position 4), and 2 ml of the suspension are inoculated and grown for ten days on both selective and non-selective PP NH_4 solid medium. At this stage growth of the clones is checked and each culture is transferred to selective medium and grown for an additional fortnight. Mitotic instability of the resistance is monitored by the apparition of dead sectors in the culture initially grown on non-selective medium. Alternatively, non-selective to selective growth test can be performed on early regenerating colonies after transformation

Rescue experiments are performed as follows: differentiated gametophores are plated on selective and non selective PP NH_4 solid medium and grown for 2-3 weeks. Regeneration of protonema from the leafy shoot is scored to asses mitotic stability and tissue specificity of the resistance.

DNA extraction from the moss Physcomitrella patens

(Modified from Rogers, S.O. and Bendich, A.J. 1988, Plant Molecular Biology Manual A6: 1 - 10). The mean yield of this protocol is around 3 to 5 µg DNA per gram fresh weight of protonematal tissue, corresponding to roughly 20% of the total amount of genomic DNA in 1 g of tissue (raw approximation). The procedure works fine with leafy gametophores too, but the yield might be lower. Most of the DNAs extracted this way were digestible with restriction enzymes without further purifications. The key point in this protocol was the grinding: switching from mortar and pestle to coffee grinder resulted in a 5 fold increase in yield.

Protocol

- Collect material from protonematal tissue grown for a week under standard conditions (medium supplemented with ammonium tartrate 0.5 g/l and glucose 5 g/l, 16 hours of light per day), and freeze it in liquid nitrogen (there is no need to blot the material on filter paper or to rinse it with water prior freezing, the fresh weight referred to hereafter is with adsorbed water).

- Cool down a standard coffee grinder (stainless steel buckett (no ceramics), diameter 7 cm, depth 3.5 cm, power around 140 W, such grinder cost around 20 SFr here, and you buy a new one when it breaks down) by grinding ca 10 g of dry ice in it (ca 30 sec).

- Mix in the coffee grinder bucket 5-10 g of plant material, 5-10 g of dry ice and grind for 30 seconds. Store the grinded material on dry ice if several samples have to be extracted in the same time. The coffee grinder bucket can be conveniently rinsed with ethanol between different samples. Store grinded tissue at –70 overnight to evaporate the dry ice: this will avoid the formation of bubbles when the grinded tissue is added to the extraction buffer

- Dispense 2 x CTAB (1ml / g Fresh W.) freshly supplemented with 20 mM ß-mercaptoethanol in 50 ml Falcon blue cap, and preheat the extraction buffer at 65^{o} C in a water bath. Preheat the 10% CTAB solution in the same time.

- Slowly add the grinded material to the tube, mix to obtain a homogenous slimy soup and incubate at 65^{o} C for 15 minutes. We have found that the yield of DNA was good even after 5 min incubation in 2 x CTAB, and that incubations longer than 20 minutes resulted in DNA degradation. Complete to a final volume of two times the weight of the sample.

- Bring the tubes back to room temperature (water bath) and add 1 volume of $CHCl_3$/isoamyl alcohol (24:1)

- Mix vigorously to obtain a stable emulsion, then centrifuge at 4000 rpm for 15 minutes (ca 2500 g in a swing-out rotor).

- Transfer the supernatant (aqueous phase) to a clean centrifuge tube and measure its volume (the SN is sometimes yellow, but this does not interfere with digestibility).

- Add 1/10 of this volume of 10% CTAB solution and mix (a transient milky precipitate appears upon the addition of 10% CTAB, mix until it disappears).

- Add 1 volume $CHCl_3$/isoamyl alcohol, mix and centrifuge for 10 minutes at 10000 g in a swing-out rotor.

- Transfer the clear supernatant to a clean centrifuge tube and measure its volume. Add one volume of CTAB precipitation buffer and leave for 30 minutes at RT°. A milky precipitate forms immediately upon the addition of precipitation buffer. If it doesn't appear, it means that the yield of the extraction is low.

- Centrifuge for 15 minutes at 10000 g in a swing-out rotor. The supernatant can be yellow, but should be completely clear at this stage. Otherwise repeat centrifugation.

- Discard the supernatant and resuspend the pellet in 400 µl HSTE (100 µL / g FW). Assist redissolution of the pellet by incubation at 50° C and if necessary with a Pipetman (can be stored overnight at 4°C).

- Transfer the solution to an Eppendorf, rinse the wall of the tubes with 100 µl of HSTE and pool in the Eppendorf. If the solution remains trouble (unsoluble polysaccharides ???), spin down the debris (10 min full speed table centrifuge) and transfer SN to a fresh tube. You can check the pellet for the presence of DNA (resuspend in TE and load on a gel), but there shouldn't be any in it.

- Precipitate DNA with 2.5 volume of absolute ethanol and leave for 30 minutes at -20° C. At this stage, the precipitate can often also be spooled. However, we haven't found any difference in the quality of spooled versus pelleted DNA..

- Centrifuge 10 minutes at 10000g in a microfuge. Discard supernatant, wash the pellet with 500 µl EtOH 70%, dry the DNA and then resuspend the pellet in 100 - 200 µl 0.1 x TE + RNAseA (20µL / g FW). Store at -20° C. Agar-like insoluble particles are occasionally found with DNA in the EtOH pellet. After complete resuspension of the DNA, centrifuge the tubes 10 min at 10000 g and discard the pellet without regrets, it doesn't contain DNA !!.

- Quantify your DNA by loading 1 to 3 µl on a gel. Use undigested lambda DNA as standards (typically 50, 150 and 250 ng). 3 ug of DNA is sufficient to detect a single copy gene with a 500 bp probe by Southern blot analysis (genome size is ca $3.5 \cdot 10^8$ base pairs per haploid genome)

Solutions

2 X CTAB

2 % CTAB (w/v)

100 mM Tris (pH 8.0)

20 mM EDTA (pH 8.0)

1.4 M NaCl

1% PVP (polyvinylpyrrolidone soluble) Mr 40000

10 % CTAB

10 % CTAB

0.7 M NaCl

CTAB Precipitation buffer

1 % CTAB

50 mM Tris (pH 8.0)

10 mM EDTA (pH 8.0)

High salt TE (HSTE)

10 mM Tris (pH 8.0)

1 mM EDTA (pH 8.0)

1 M NaCl

0.1 X TE

1.0 mM Tris (pH 8.0)

0.1 mM EDTA (pH 8.0)

Additional notes

Don't start with lyophilized material since it does not increase yield and result in more degraded DNA.

Never try to purify CTAB-extracted DNA with phenol, or you won't be able to digest your DNA anymore (unknown reasons, but observed in other hands too).

Uncut DNA extracted this way has a MW above 20 to 30 kb. However, this may be typical for *P. patens*, since uncut DNA extracted with the same procedure from tobacco, sugar beet or *Portulaca* has a much higher molecular weight (above 50 kb).

This protocol can easily be scaled up or down.

The titer of the DNA solutions ranges from 100 to 300 ng/µl. The solution is rather viscous and tRNA contamination is not too important, allowing OD 260 - 280 nm quantification to be performed (I usually quantify on EtBr gel, using 50, 100 150 200 250 ng of uncut lambda DNA as standards). These DNA have been routinely digestible with most restriction enzymes. Digestions are always conducted in a final volume corresponding to 10 times the initial volume of DNA for 3 to 4 hours with 3 to 5 units of enzyme per µg DNA. Problems have been encountered with restriction enzymes sensitive to cytosine methylation.

PCR reactions have been successfully performed on such DNA

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