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A Simple, Handheld Apparatus for Generating Small Volumes of $^{15}N_2$ from ^{15}N-Labeled Ammonium Salts

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Generating $^{15}N_2$ gas is an important technique for directly tracing the rate of nitrogen fixation and the fate of these ^{15}N-labeled metabolites within the organism or ecosystem (1–4). For these experiments $^{15}N_2$ gas is usually evolved from ^{15}N-labeled $(NH_4)_2SO_4$ or NH_4Cl by reaction with strong hypobromide solution. However, other ^{15}N-labeled oxides can also be produced and these may be directly assimilated as a N source by the organism and thus can cause serious errors in interpreting $^{15}N_2$ labeling experiments. To avoid these contaminant oxides the newly evolved $^{15}N_2$ needs to be sparged through various solutions while avoiding dilution with atmospheric $^{14}N_2$, an operation that usually requires specialist glassware and vacuum pumps (3). As an alternative, a handheld apparatus is described here that can generate small volumes (5 to 500 cc^3) of contaminant-free $^{15}N_2$ without other glassware or vacuum lines. The $^{15}N_2$ gas evolved from this apparatus was applied to wheat plants grown under sterile conditions and no enrichments (above background) of the shoot tissue could be detected, indicating that the gas was free of ^{15}N oxide contaminants.

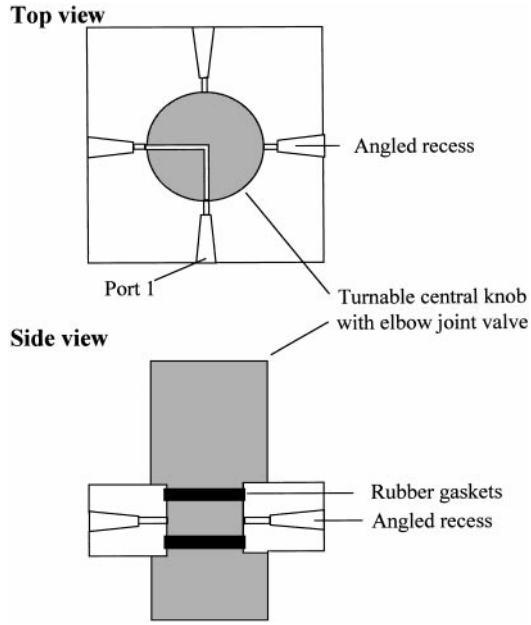

FIG. 1. Top and side view of handheld apparatus for generation of $^{15}N_2$ gas from ^{15}N-labeled ammonium salts. The top view (width 6 cm) shows the four recessed ports for insertion of syringes and the central turnable knob (shaded area) with a 90° elbow bend allowing adjacent ports to be connected. The four ports can be labeled 1 to 4, clockwise direction. The side view of the apparatus shows the placement of the access ports and the rubber gaskets.

The $^{15}N_2$ gas was generated using the handheld apparatus as shown in Fig. 1 (top view and side views). The basic principles of the design include the four access ports and the central turnable knob with a 90° angle bend allowing adjacent ports to be connected. The ports are beveled to allow a tight fit for the end portion of common syringe cylinders (Fig. 1, upper panel) and the central knob has two rubber O rings to form a gas tight seal (Fig. 1, lower panel). The entire body of the apparatus was made from clear Perspex and was found to be resistant to the chemicals used in

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the subsequent reactions. Bergersen (3) describes all the preparations for solutions required for generating $^{15}N_2$ from ^{15}N-labeled $(NH_4)_2SO_4$ and the subsequent procedures for ensuring that the gas is free from contaminating oxides. The following stock solutions were prepared (nanopure water was used throughout and room temperature unless specified): A, 2 M (^{15}N-labeled $(NH_4)_2SO_4$ (80%, atom percent, Cambridge Isotope Laboratories, MA); B, 12 M LiOBr (See Bergerson (3) for preparation and storage); C, 20 mM $KMnO_4$ acidified with 1% v/v concentrated HCl; and D, distilled water acidified with 1% v/v concentrated HCl. To generate 50 cc^3 of 80% enriched $^{15}N_2$, 1 mL of A (containing 2×10^{-3} mol ^{15}N is drawn up into a 1-mL syringe, 30 mL of B is drawn up into a 100-mL syringe, 30 mL of C is drawn up into a 100-mL syringe, and 30-mL of D is drawn up into a 100-mL syringe. Care is taken to ensure that no extra air is introduced into each syringe and excess air is expelled. Syringes A, B, C, and D are fitted into the beveled ports, taking care to rotate the central knob to a neutral position so that the solutions cannot come into contact. The apparatus should be held in the hand so that syringe A (port 1) is horizontal, syringe B (port 2) is vertical, and the handle of the knob faces the user. The reaction sequence is initiated by turning the central knob so that port 1 is connected to port 2. Solution A is pushed firmly, by hand, into Solution B, $^{15}N_2$ is evolved and by maintaining a constant pressure on the plunger of syringe 1 the plunger of syringe 2 is pushed back along the carriage accommodating about 50 cc^3 of $^{15}N_2$ gas. The solutions are thoroughly mixed by shaking the apparatus. It is important that LiOBr is in excess and the characteristic yellow color of Solution B should be visible in syringe 2. (If the mixture of Solutions 1 and 2 is clear, it is possible to introduce more of Solution 2 by adjusting the central knob to a neutral position, removing syringe 1, and adding another 100-mL syringe filled with 10 mL LiOBr). The apparatus is rotated so that syringe 2 (port 2) is now horizontal and syringe 3 (port 3) is vertical. Adjust the central knob so that ports 2 and 3 are connected and adjust the body of the apparatus so that the gas is sparged through Solution C. Take care to avoid contamination with the $KMnO_4$. Repeat this procedure to sparge the gas through Solution D. A syringe needle is prepared and syringe 4 is removed from the body of the apparatus and the needle is fitted. Store the gas for short periods (hours) with the syringe vertical (needle down) leaving some of Solution D to form a seal.

The $^{15}N_2$ gas generated via this apparatus was applied to N-starved wheat plants grown within a confined head space (5). After 70 h the shoot tissue was analyzed for the enrichment of ^{15}N via assessment of the isotopic ratios using a MicroMass 903 mass spectrometer fitted with three bucket collectors for masses 28, 29, and 30. The shoot tissue had a $0.3636 \pm 0.006\%$ enrichment of ^{15}N, which was not significantly more than that of a control plant not exposed to gas ($^{15}N_2$, $0.3669 \pm 0.007\%$) nor significantly different from the natural abundance published by Rennie and Rennie (Ref. 6, $0.3666 \pm 0.004\%$). A positive enrichment control where ^{15}N-labeled $(NH_4)_2SO_4$ salt was added direct to the wheat root solution produced a rapid and significant enrichment within the 70-h experiment (42% enrichment N labeled with ^{15}N). As our mass spectrometer can only accept analyses of liquid samples the purity of the $^{15}N_2$ gas produced by the apparatus could not be directly measured in our instrument. To test for generation and purity of the N_2 gas produced by the apparatus the free-living diazotroph *Azospirillum brasilense* was grown for 1 week under under $^{15}N_2$-fixing conditions (7) in an enclosed flask where the headspace was evacuated and flushed twice with argon and replaced with gas generated from the apparatus. *A. brasilense* cells grown under these N_2-fixing conditions were harvested and analyzed as per the plant material. The ^{15}N enrichments were 75% ^{15}N derived from the atmosphere, an enrichment that is near maximal (i.e., 80%) and indicates that the gas produced from the apparatus was not diluted with large volumes of atmospheric $^{14}N_2$. Therefore, the $^{15}N_2$ gas generated by this apparatus gas can be considered biologically inert (except for the activity of nitrogenase) and free of N oxide contaminants. Furthermore the $^{15}N_2$ gas was only marginally diluted by atmospheric N_2 during its generation. In summary, this handheld apparatus avoids most of the equipment normally required for the generation of $^{15}N_2$ from ammonium salts. The apparatus may be recommended for small-scale experiments in the laboratory or in field situations where the generation of $^{15}N_2$ for isotope tracing experiments presents the most direct and artifact-free method for assessing short periods of N_2 fixation.

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High-Throughput Polymerase Chain Reaction Cleanup in Microtiter Format

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In recent years the amount of sequencing has increased tremendously, especially due to the introduction of automated DNA sequencing. PCR[2] is today a principal method for the synthesis of templates for DNA sequencing. Consequently, the need for high-throughput PCR cleanup for template preparation has increased. Although many methods have been reported, there is still a need for improved methodology in particular with respect to speed, labor, cost efficiency, and quality. The method reported here is a further development of our previously described method (3), now adapted to the 96-microtiter format, allowing high-throughput purification.

Diatomaceous earth (DE; D-5384, Sigma). DE is used as the DNA-binding matrix. It is prepared by suspension to 50 mg/ml in water and left to sediment for 2 h. The unsettled fines are removed by aspiration leaving the sediment intact. The washing is repeated once and reconstituted to the original volume with water (3).

Preparation of the arrayed spun columns. Pipette tips (0–300 μl; Dandidag pipetter Aps, DK) are placed in a 96-microtiter format solid holder (2 cm high containing 96, 3-mm-diameter holes). The holder is placed on top of a 1.2 ml Deep Well Plate (Advanced Biotechnologies Ltd, UK) which acts as support and reservoir for flow-troughs. A single 1-mm solid-glass bead (KeboLab, Sweden) is added to each tip, by use of vacuum and a 12-channel Immunowasher (Nunc, Denmark). The glass beads act as frits and are fixed in the conical tips by centrifugation at 1000*g* for 1 min in a Sigma 4K15 centrifuge equiped with a 9100/9158 rotor (used throughout this method). Fifty microliters of 50 mg/ml DE are added to each column by use of a multichannel pipette and allowed to sediment for at least 15 min.

DNA purification. The prepared columns are centrifugated 5 min at 6000*g* to drain. An aliquot of 100 μl binding buffer (5 M guanidinium thiocyanate, 10 mM EDTA, 50 mM Tris-HCl, pH 6.8) is added to each column. Note that compared to previously published methods for purification of DNA by DE (2, 3), this binding buffer has a lower pH value, allowing efficient binding of smaller DNA fragments (>100 bp). Then the PCR solutions (25–50 μl) are loaded and centrifugated for 10 min at 6000*g* to allow binding and draining. Primers and other contaminants are removed by adding 200 μl 80% isopropanol followed by centrifugation for 10 min at 6000*g*. A 96-well PCR plate (Techne, UK) is inserted between the columns and the supportive 1.2 ml Deep Well Plate. The bound DNA is eluted by adding 25–50 μl Te (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) followed by centrifugation for 10 min at 6000*g*. The concentration of the eluted DNA depends on the preceding PCR. In general, we obtain 20–100 ng/μl, a concentration suitable for cycling sequencing.

This method can simultaneously purify 2 × 96 DNA templates in less than an hour at a price of approximately 1.80 USD per 96 samples. As judged by agarose gel electrophoresis, the recovery is routinely >80% of the PCR product applied to the spun columns (data not shown). The failure rate is 0–1 out of 96 spun columns and is typically due to clogging of the failed column. The quality of the DNA purified by this method was tested by dye-terminator cycle sequencing (BigDye terminator cycle sequencing reaction kit, PE Applied Biosystems, U.S.A.) using an ABI Prism 310. A representative electropherogram is shown in Fig. 1, illustrating low background and sharp peaks sufficient to allow sequencing of 400 bases. We have used this method routinely for sequencing a 278-bp PCR fragment from clones isolated in a phage display library approach. Unambiguous sequence data could be obtained for more than 95% of the tested clones.

High-throughput purification protocols for purifica-

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[2] Abbreviations used: PCR, polymerase chain reaction; DE, diatomaceous earth.

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