

## Abstract

**Purpose:** To develop a method of stabilising the purified, reconstituted multi drug efflux pump P-glycoprotein (P-gp or ABC1) to enable storage at temperatures above what was previously possible enabling the design of a high throughput assay.

**Methods:** The purification of P-gp was altered to include the substitution of glycerol for trehalose/maltose at the point of elution. Hexahistidine tagged P-gp was expressed in *Tricopulisia ni* (High Five) cells and cell membranes were isolated by centrifugation. Membrane proteins were solubilised and P-gp was purified using metal affinity chromatography before being reconstituted into proteoliposomes. These were then slowly frozen to -80°C in vials with stoppers and freeze-dried under vacuum of 0.011mBar for 72 hours. Vials were then sealed whilst still under vacuum and the freeze-dried product was stored ready for assessment of ATPase activity.

**Results:** The ATPase activity of conventionally purified P-gp was assessed following storage at temperatures between -80°C and +20°C for up to 250 days and was seen to decrease rapidly at all temperatures except -80°C. Freeze drying of P-gp eluted in glycerol-based buffer resulted in 95% loss of ATPase activity on rehydration. Trehalose and maltose were then explored as possible lyoprotectants. It was demonstrated prior to purification of P-gp in the presence of these disaccharides that there were no serious deleterious effects on ATPase function at concentrations up to 300mM. P-gp was subsequently purified with an altered procedure, namely the substitution of 20% v/v glycerol for 20% w/v trehalose/maltose just prior to elution. Trehalose was found to be considerably more effective than maltose in protecting P-gp in the dried state. After 60 days trehalose samples stored at +4°C retained 73±12% (n=4) ATPase activity, those at +20°C retained 64±14% (n=4) and those at +37°C retained 37±4% (n=4).

**Conclusion:** Purified, reconstituted P-gp can, following optimisation of the purification procedure via substitution of glycerol for trehalose, be successfully stabilised by freeze drying over a range of temperatures.

## Methods and apparatus

P-gp was purified and freeze dried according to recently published and patented methods [1,2 and 3]. Briefly hexa-histidine tagged P-gp was purified using metal affinity chromatography, reconstituted into proteoliposomes and freeze dried at 0.011mBar 72h in the presence of trehalose.

All freeze drying was performed using a MartinChrist Alpha 2-4. Vials were sealed under vacuum and stored in temperature controlled environments for the purposes of storage testing.

Functional protein ATPase activity was assayed using colorimetric detection of inorganic phosphate (Pi).

Michaelis-Menten kinetic analysis was performed on GraphPad Prism v3&4 (US).

## Need for stabilisation of P-gp

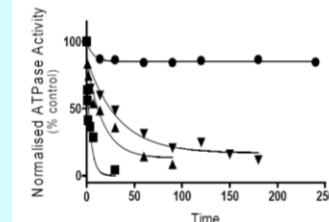


Fig.1 Loss of ATPase activity of conventional P-gp over time

Fig. 1 shows the ATPase activity of reconstituted ABCB1 stored at a number of temperatures between -80 and +20°C for up to 250 days. All values of ATP hydrolysis were obtained in the presence of nicardipine and normalised against the activity prior to storage (100%). The ATPase activity decreased in a time dependent fashion at storage temperatures other than -80°C and measurements were ceased once activity was less than 10% of the initial value.

## Assessment of IMAC purification of P-gp

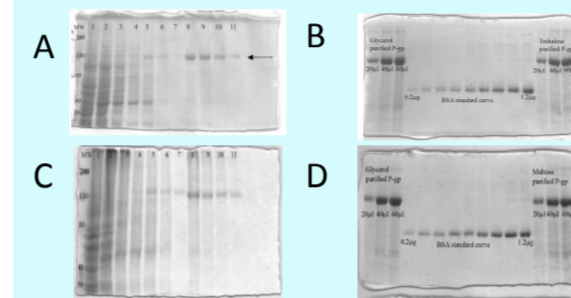


Fig. 2 Purity (A and C) and yield (B and D) of P-gp obtained using alternative disaccharide buffers. The purification of His6-tagged ABCB1 by IMAC was monitored using SDS-PAGE with Coomassie-Blue staining.

P-gp was purified using progressive washes of imidazole in buffer containing lipids and detergent with final purity being >90% (Figs 2.A and C). In order to determine yield for calculating specific protein ATPase activity (μM Pi/min/mg P-gp) volumes of reconstituted P-gp were run on SDS-PAGE alongside a BSA standard curve (Fig 2.B and D). P-gp yield could then be calculated using densitometric analysis. Using 50mg of High-5 insect cell crude membranes gave ABCB1 yields of 115±41 μg, 108±29 μg or 116±10 μg for buffers containing glycerol, trehalose or maltose respectively.

## ATPase activity of pure P-gp

The basal ATPase activity of purified trehalose (V<sub>max</sub> = 0.52±0.14 μmol Pi/min/mg) or maltose (0.50±0.14 μmol Pi/min/mg) containing buffers was not significantly altered from the values obtained using a standard glycerol based procedure. Additionally nicardipine stimulated (drug stimulated) V<sub>max</sub> was similarly unaffected (data not shown).

## Freeze drying of pure, reconstituted P-gp

Conventionally (glycerol) purified P-gp does not withstand freeze drying with less than 5% of pre-freeze dried ATPase activity remaining (Fig. 3.A). However on modification of the purification technology to include maltose or trehalose approx. 69% and 83% activity remained respectively (Figs. 3.B and 3.C).

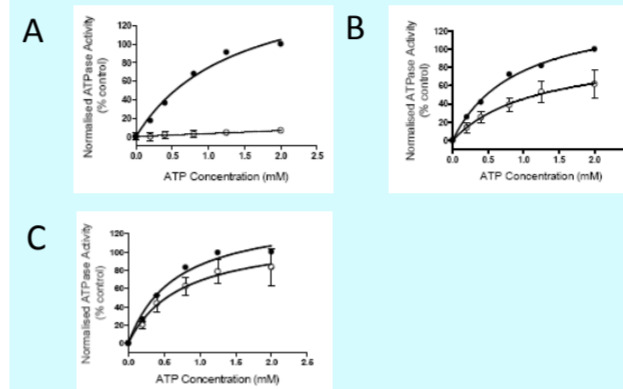


Fig. 3 Pre- and post-freeze drying drug stimulated ATPase activity of convention glycerol purified P-gp (A) and modified maltose (B) and trehalose (C) purified P-gp.

## Long term stability of freeze dried P-gp

In order to present an truly effective solution to the instability of purified P-gp proteoliposomes and facilitate the development of a high throughput screen long term testing was carried out over a range of temperatures. Sealed freeze dried vials were stored at 4°C, 20°C and 37°C for up to 150 days and ATPase activity was measured periodically. Trehalose samples tested after 60 days storage at +4°C displayed 73±12% (n=4) of their pre-freeze drying ATPase activity whilst those at +20°C displayed 64±14% (n=4) and those at 37°C only 37±4% (n=4). Prolongation of storage to 150 days was associated with further loss in ATPase activity, although the magnitude of reduction was considerably less (Fig. 4). Maltose samples lost activity rapidly during storage (data not shown).

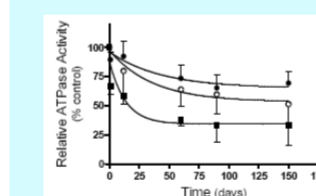


Fig. 4. Stability testing of freeze dried P-gp stored over 150 days at 4°C (●), 20°C (○) and 37°C (■).

## Validation of assay against clinically relevant compounds

The ability to determine Michaelis-Menten kinetics has been shown (above) however this is not the full extent of data and information which can be obtained with this assay.

By altering the format of the assay pharmacological parameters such as potency of drug interaction (EC<sub>50</sub>), potency of inhibition (IC<sub>50</sub>) and identification of true inhibitors as distinct from substrates can be determined.

We have demonstrated our ability to measure the differing potencies of drug interaction with several compounds namely: nicardipine, a well known and potent P-gp modulator; prazosin, frequently used as a P-gp probe; vinblastine, an anti-cancer chemotherapeutic agent and verapamil, a calcium channel blocker frequently cited as a P-gp 'inhibitor' (see Figs 5A – 5D).

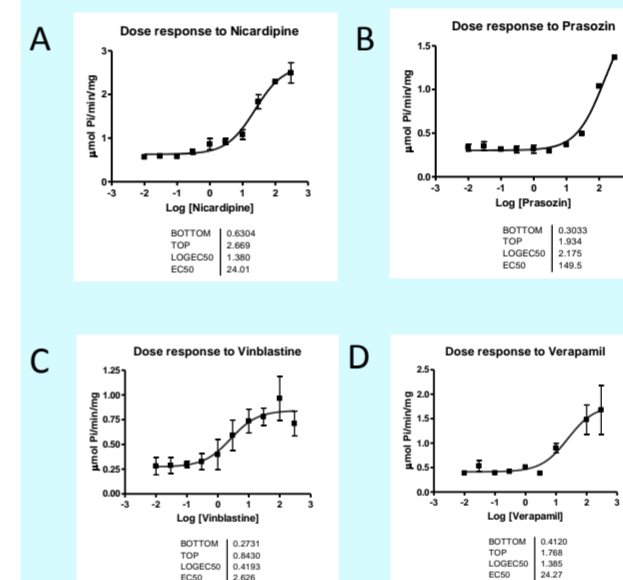


Fig. 5 Dose response curves yielding pharmacological data regarding drug-P-gp interaction in a dose dependent manner. EC<sub>50</sub>s determined for nicardipine, prazosin, vinblastine and verapamil.

## Discussion

We have presented a novel method of purifying P-gp, a clinically relevant multi-drug efflux pump capable of transporting a wide and disparate range of drugs, which allows for successful stabilisation via freeze drying. The dried product can be stored over a range of temperatures for up to 6 months without undue loss of functionality as measured by ATPase activity. On re-hydration the purified proteoliposomes allow for the identification and pharmacological characterisation of compounds which interact with P-gp without interference from other drug transporters or ATPases.

We have demonstrated that this assay is robust, flexible and reproducible and have provided data to show its validation against industry accepted standard substrates and probes for P-gp function [4].

## References

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