capillary endothelial cells of the brain, the canalicular domain of the hepatocyte, the brush border of the proximal renal tubule, the placenta and the testis.

Human *ABCB1* gene is located on chromosome 7q21, and the gene product is an integral membrane protein consisting of 1280 amino acid residues (Fig. 8). ABCB1 is composed of two homologous halves each of which consist of an N-terminal, hydrophobic, membrane-associated domain (approximately 250 amino acid residues) and a C-terminal, hydrophilic nucleotide binding fold (approximately 300 amino acid residues). The plasma membrane associated domains in the two halves of ABCB1 each consist of six transmembrane domains, which are followed by an intracellular ATP-binding cassette. There are two ATP-binding cassettes in one molecule of the ABCB1 protein. Those ATP-binding cassettes are functionally non-identical, but essential for the transport function of ABCB1 (Senior *et al.*, 1995; Sauna and Ambudkar, 2000). Since ABCB1 is an ATP-dependent active transporter, drug transport is coupled with ATP hydrolysis. The ATPase activity can be determined by measuring inorganic phosphate liberation (Carter and Karl, 1982; Sarkadi *et al.*, 1992). This property is advantageous to screen the function and substrate specificity of ABCB1 (Garrigues *et al.*, 2002; Onishi *et al.*, 2003).

V-2. High-speed screening system to measure ABCB1 ATPase activity

We have recently developed a high-speed screening system using 96-well plates to analyze the substrate specificity of ABCB1 and its genetic variants (Onishi *et al.*, 2003; Ishikawa *et al.*, 2004). ABCB1 has been expressed in insect cells. Fig. 9 schematically demonstrates the protocol of ATPase activity assay. Briefly, cell membranes expressing ABCB1 (2 μg of membrane protein per each well) are suspended in 10 μl of the incubation medium containing 50 mM Tris-Mes (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM potassium chloride, 5 mM sodium azide, 2 mM ouabain. This medium is mixed with 10 μl of a test compound solution and then pre-incubated at 37°C for 3min. The ATPase reaction is started by adding 20 μl of 4 mM ATP/Mg solution to the reaction mixture and the incubation was maintained at 37°C for 30 min. The reaction is

stopped by the addition of 20 μl of 5% trichloroacetic acid and subsequently with 42 μl of "solution A" and "solution B" (see Fig. 9 for details). Thereafter, 120 μl of "solution C" is added to the mixture. These mixing processes are automatically carried out in the HALCS-I system (BioTec Co. Ltd., Tokyo Japan). The absorbance of each reaction mixture in the 96-well plates is photometrically measured at a wavelength of 625 nm in a Multiskan JX system (Dainippon Pharmaceuticals Co., Osaka, Japan). The amount of liberated phosphate can be quantified based on the calibration line established with inorganic phosphate standards. In addition, each 96-well plate should contain a positive control, in which Sf9 cell membranes are incubated with 10 μM verapamil. Fig. 10A shows a Michaelis-Menten-type relationship between ABCB1 ATPase activity and verapamil concentration.

V-3 Genetic polymorphisms of ABCB1 vs. ATPase activity

To date, genetic variations of the human *ABCB1* gene have been extensively studied (Kerb *et al.*, 2001; Sparreboom *et al.*, 2003 for recent reviews). Fig. 8 depicts hitherto identified non-synonymous polymorphisms in the ABCB1 protein. Several preclinical and clinical studies have provided evidence for the naturally occurring polymorphisms in ABCB1 and their effects on drug absorption, distribution and elimination. However, quantitative studies are required to precisely evaluate functional changes associated with such genetic polymorphisms of ABCB1. For this purpose, we have prepared several variant forms (i.e., N183S, S400N, R492C, R669C, I849M, A893T, M986V, A999T, P1051A, and G1063A) by site-directed mutagenesis. These variants of ABCB1 were then expressed it in Sf9 cells using the pFASTBAC1 vector and recombinant baculoviruses, as described previously (Ishikawa *et al.* 2004; Sakurai *et al.*, manuscript inpreparation). ABCB1 variant proteins expressed in Sf9 cell membranes are detected by the western blot method using the C219 monoclonal antibody. Using membranes prepared from insect cells expressing those ABCB1 variants, ATPase activity has been measured in the presence of verapamil at various concentrations. Fig. 10B summarizes kinetic parameters (Km and Vmax values) observed with verapamil for those variant forms as well as the wild type of ABCB1. Km and Vmax

values for verapamil vary to some extents among variants. The Vmax values of those variants were normalized to that of the wild type by referring to the intensity of each variant protein on the immunoblotting as shown in Fig. 4B. Kinetic parameters of non-synonymous polymorphisms of ABCB1 observed with different substrates will be reported elsewhere.

VI. Quantitative SAR analysis to evaluate the substrate specificity of ABCB1

To understand the impact of non-synonymous polymorphisms on the function, it is critically important to quantitatively analyze the functional difference among such variants. We have developed a method of quantitative structure-activity relationship (SAR) analysis. Using the high-speed screening system, we first measure ABCB1 ATPase activity toward a total of 41 different therapeutic drugs and compounds. The tested compounds are classified into seven groups, *i.e.*, A, neurotransmitters; B, Ca^{2+} channel blockers, C, steroids; D, potassium channel modulators; D, non-steroidal anti-inflammatory drugs (NSAIDs); F, anti-cancer drugs; and G, miscellaneous. Fig. 11 demonstrates the effects of those test compounds on ABCB1 ATPase activity. The concentration of test compounds was 10 μM in the measurement, and the data are expressed as relative values as compared with the ATPase activity measured with 10 μM verapamil. Among 41 different therapeutic drugs and compounds tested in this study, Ca^{2+} channel blockers, such as verapamil (B-1), bepridil (B-4), fendiline (B-5), prenylamine (B-6), nicardipine (B-7), and FK506 (G-4) stimulated the ATPase activity. At the concentration of 100 μM, paclitaxel (F-5), doxorubicin (F-7), and quinidine (G-1) have more significantly stimulated the ABCB1 ATPase activity, whereas the extent of ATPase stimulation was relatively smaller than that of Ca^{2+} channel blockers (data not shown).

Chemical fragmentation codes are practical and useful to describe the chemical structures of a variety of substrates and non-substrates for ABCB1. Derwent Information, Ltd., developed this structure-indexing language suitable for describing chemical patents. Markush TOPFRAG is the software that generates the chemical fragment codes from chemical structure information.

We use the Markush TOPFRAG to generate chemical fragmentation codes for each compound tested in Fig. 11. The multiple linear regression analysis is carried out to gain a relationship between the ABCB1 ATPase activity and the chemical fragmentation codes thus generated. Thereby we identify several sets of chemical fragmentation codes related to the substrate specificity of ABCB1. For example, based on the data shown in Fig. 11, a total of six best-fitting models were created. Table 1 summarizes the contents of those multiple linear regression analysis models, and Table 2 provides explanations for chemical fragmentation codes generated in the analysis. These results demonstrate that the moieties represented by the chemical fragmentation codes of J581, G100, and M331 positively contribute to the ATPase activity, whereas those of M531 and F014 have negative contributions. Among those chemical fragmentation codes, J581 has the greatest contribution (Table 1), suggesting that an oxo group bonded to an aliphatic carbon (Table 2) is an important moiety for the recognition and/or transport by the ABCB1 protein. In addition, it is suggested that unfused aromatic ring (G100) and straight carbon chain (M331) are important chemical moieties for the substrate specificity of ABCB1.

The uniqueness of this approach resides in the facts that ABCB1 ATPase activity is described as a linear combination of chemical fragmentation codes and that the coefficient for each chemical fragment code reflects the extent of the contribution of a specific chemical moiety to the ATPase activity. The point in the catalytic cycle at which substrate-binding takes place, and details of how ATP hydrolysis drives transport may be critical for understanding the mechanism of substrate specificity. This quantitative SAR analysis can be applied for each variant form of ABCB1 to gain more insight into the effect of non-synonymous polymorphisms on the substrate specificity of ABCB1.

VII. SNP array to detect genetic polymorphisms of ABC transporters

It is estimated that about 3 million SNPs are derived from comparison of genomic sequence from individuals among several populations. These variants may be used as an important tool in association studies or linkage disequilibrium mapping to elucidate the

genetic foundation of multi-factorial disorders and individual differences in drug response. However, it is not realistic to analyze all SNPs for each patient, since the number of SNPs is so large and each SNP must ultimately be evaluated in terms of functionality. We therefore at first validate SNPs of drug transporters as well as drug metabolizing enzymes from functional point of view. Through functional validation, the number of SNPs can be significantly reduced. A limited set of SNPs that are closely related with pharmacokinetics will be analyzed by a cost-effective and automated analytical technology. We have recently developed a "SNP array" method to meet such practical and clinical needs. Based on our functional assay data as well as currently available SNP databases on drug transporter genes, SNP-specific probes are carefully designed and spotted on the epoxy-activated surface of glass plates by means of the GENESHOT system (NGK Insulators, Ltd. Japan). The size of each probe spot is 50 μm in diameter. Each SNP is detected by one set of probes (usually five oligo-nucleotide probes) that have different stringencies of hybridization with DNA samples.

Fig. 12 shows a flowchart of the SNP detection process, i.e., DNA sample preparation, multiplex PCR, hybridization on the SNP array, and fluorescence signal measurement. DNA samples for SNP array analysis is amplified by multiplex PCR with biotin-linked primers and genomic DNA extracted from human white blood cells. The multiplex PCR conditions are as follows: 94°C for 5 min, 35 cycles of 95 °C for 30 sec, 62.5°C for 30 sec, and 72°C for 30 sec, and finally 72°C for 2min. The resulting PCR product (2.5 μl) is subsequently mixed with 2.5 μl of 0.6 M NaOH. Hybridization with SNP probes is performed in 20 μl of hybridization buffer solution consisting of 200 mM citrate/phosphate (pH 6.0), 2% SDS, 750 mM NaCl and 0.1% NaN_3. The hybridization is maintained at 55°C overnight. After hybridization buffer solution and excess of the PCR product are removed by rinse, the SNP array is treated with Cy5-linked streptavidin in the TNB solution (0.1 M Tris/HCl, pH 7.6, 0.15 M NaCl, and 0.5% NEN blocking reagent) at room temperature for 30 min. Since biotin-labeled primers are used in the multiplex PCR, the hybridized PCR product can be conjugated with streptavidin. Thus, Cy5-linked streptavidin enables fluorescence detection of the hybridized PCR product with excitation light at 633 nm (He/Ne

the hybridized PCR product with excitation light at 633 nm (He/Ne laser) or 647 nm (Ar/Kr laser). Fig. 13 shows the result of SNP array detection, where non-synonymous polymorphisms of ABCG2, i.e., Q126stop and Q141K, were analyzed. Homo- and hetero- alleles of 141-Gln (Q) and 141-Lys (K) in human samples were detected and clearly distinguished with this SNP array.

Recently Imai *et al.* (2002) identified three allelic variants in the ABCG2 gene, of which two were non-synonymous SNPs (V12M and Q141K) and the third was a splice variant with deletion of nucleotides 944-949 that lacks Ala-315 and Thr-316 (Δ315-6). Compared with wild-type transfected cells, the ABCG2 Q141K variant-transfected cells showed a low level of drug resistance that is associated with decreased protein expression. The SNP (Q141K) was postulated to cause increased sensitivity of normal cells to anticancer agents that are ABCG2 substrates such as SN-38. Moreover, a novel SNP in exon 4, 376C>T, substituting stop codon for Gln-126 was found in Japanese population with an allelic frequency of 2.4% (Honjo et al., 2002). It has been postulated that the 376C>T SNP may have higher impact than 421C>A polymorphism causing Q141K, because active ABCG2 protein will not be synthesized from the variant allele.

VIII. Concluding remarks

Pharmacogenomics is recognized increasingly important for predicting pharmacokinetic profiles and/or adverse reactions of drugs (Kalow *et al.*, 2001). Drug transporters as well as drug-metabolizing enzymes play pivotal roles in determining the pharmacokinetic profiles of drugs (i.e., drug absorption, distribution, metabolism and elimination, as well as drug concentration at the target site). The effects of drug transporters on the pharmacokinetic profile of a drug depend on their expression and functionality. There are an increasing number of literatures that address genetic polymorphisms of drug transporters. However, it is also true that there is still considerable discrepancy among hitherto reported results. Functional analysis of the polymorphism of drug transporters is one of such important approaches that provide clear insight into the biochemical significance of genetic

polymorphisms (Ishikawa *et al.*, 2004).

This chapter conveys a new strategy of analyzing the relationship between the substrate specificity of ABC transporters (e.g., ABCG2 and ABCB1) and the chemical structure of substrates. This approach is applicable for the functional analysis of genetic polymorphisms of ABC transporters. The effect of SNPs on the transport activity may depend on substrates tested, and therefore the functional analysis of SNPs using a wide variety of substrates is of great interest. One amino acid substitution can alter interactions between the active site of an ABC transporter and substrate molecules. Therefore, it is critically important to quantitatively analyze and evaluate such structure-related interactions. In this context, the new SAR analysis using chemical fragmentation codes will provide a powerful tool to quantify the impact of genetic polymorphisms on the function of ABC transporters. Furthermore, as demonstrated in this chapter, the SNP array method is a simple and practical tool to detect SNPs that can affect the function and/or expression of drug transporters. Combination of SNP detection with functional evaluations will provide clear insights into the molecular mechanisms underlying individual differences in the drug response of patients.

IX. Acknowledgments

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Figure Legends

Fig. 1. Schematic illustration of drug actions in the target cell. Drug transporters play a significant role in the absorption and excretion of drugs, and thereby modulate pharmacological and toxic effects. Phase I, II, and III systems of drug metabolism and excretion are indicated.

Fig. 2. Schematic diagram of the pNNK8 transfer vector for the expression of human ABCG2 in insect cells.

Fig. 3. Procedure of plasma membrane vesicle preparation from insect cells.

Fig. 4. Detection and quantitative analysis of human ABCG2 expressed in the plasma membrane of insect cells. A, coomassie staining and immunoblot analyses of membrane proteins. Plasma membrane proteins (2 μg) were separated by SDS-PAGE (10% acrylamide gel), and ABCG2 was detected by immunobloting. B, quantitative analysis of ABCG2 protein in the plasma membrane preparation. Different amounts of plasma membrane proteins (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 μg) were subjected to SDS-PAGE and ABCG2 protein was detected by immunobloting. The signal intensity of immunoblots is expressed as a function of the logarithmic value of the amount of membrane proteins applied to SDS-PAGE.

Fig. 5. High-speed assay of ATP-dependent MTX transport mediated by ABCG2 in plasma membrane vesicles. A, procedure of the vesicle transport assay using 96-well MultiScreen plates. B, high-speed screening system (BioTec EDR384S) used for the vesicle transport assay.

Fig. 6. Time courses of MTX transport in plasma membrane vesicles prepared from insect cells infected with ABCG2-virus. The inset shows the MTX transport in plasma membrane vesicles prepared from insect cells infected with ABCG2-virus and mock-virus. Transport

was measured for 20 min in the presence and absence of ATP.

Fig. 7. Non-synonymous polymorphisms of human ABCG2. A, schematic illustration of the structure of ABCG2 protein and the locations of amino acid changes. B, the effect of non-synonymous polymorphisms on MTX transport activity of human ABCG2. R482G and R482T are acquired mutations. MTX transport was measured as described in Fig. 6.

Fig. 8. Non-synonymous polymorphisms of human ABCB1. A, schematic illustration for the structure of ABCB1 protein and indicating locations of amino acid changes. G185V is an acquired mutation. The molecular structure of ABCB1 is modified from Gottesman, M.M., and Pastan, I. (1988). ABC, ATP-binding cassette.

Fig. 9. Schematic diagram of high-speed screening of ABCB1 ATPase activity.

Fig. 10. The ATPase activity of ABCB1 and its variants. A, verapamil-enhanced ATPase activity of ABCB1 measured in the plasma membrane prepared from Sf9 cells. The plasma membrane was incubated with verapamil at different concentrations (0, 1, 2, 5, 10, 20, 25, and 50 μM). Closed circles, ABCB1-expressing Sf9 cells; open circles, control Sf9 cells. B, the effect of non-synonymous polymorphisms on the ATPase activity. Verapamil-enhanced ATPase activity was measured under the same condition described in Fig. 9. For each variant form of ABCB1, Km and Vmax values were calculated from Lineweaver-Burk plots by referring to the immunoblot intensity of plasma membrane preparations.

Fig. 11. The effect of therapeutic drugs and compounds on ABCB1 ATPase activity. The ATPase activity was measured in the presence of 10 μM of a test compound. All the activities are expressed as relative values ± S.D. as compared with the activity measured with 10 μM verapamil (100%). The tested drugs and compounds are: glycine (A-1), glutamic acid (A-2), dopamine (A-3), norepinephrine (A-4), epinephrine (A-5), γ-aminobutyric acid

(A-6), histamine (A-7), serotonin (A-8), melatonin (A-9), verapamil (B-1), nifedipine (B-2), diltiazem (B-3), bepridil (B-4), fendiline (B-5), prenylamine (B-6), nicardipine (B-7), dexamethasone (C-1), betamethasone (C-2), prednisolone (C-3), cortisone (C-4), nicorandil (D-1), pinacidil (D-2), acetylsalicylic acid (E-1), indomethacin (E-2), acemetacin (E-3), ibuprofen (E-4), naproxen (E-5), mepirizole (E-6), vinblastine (F-1), etoposide (F-2), actinomycin D (F-3), daunorubicin (F-4), paclitaxel (F-5), methotrexate (F-6), doxorubicin (F-7), 5-fluorouracil (F-8), quinidine (G-1), *p*-aminohippuric acid (G-2), penicillin G (G-3), FK506 (G-4), and novobiocin (G-5).

Fig. 12 SNP detection by the SNP array method. The detection process includes DNA sample preparation, multiplex PCR, hybridization on the SNP array, and fluorescence signal measurement.

Fig. 13. Detection of non-synonymous polymorphisms (Q126stop and Q141K) of ABCG2 with the SNP array.

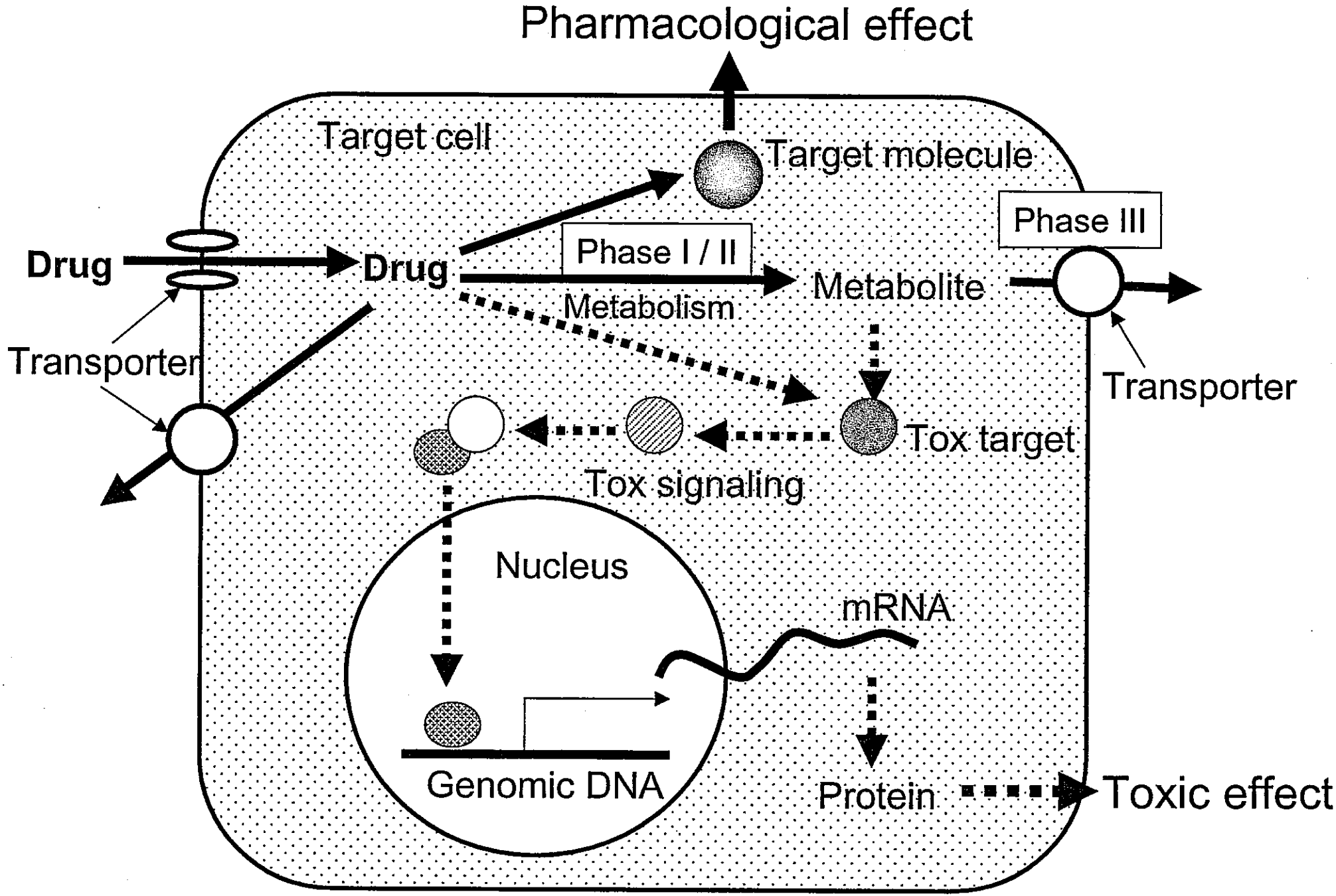


Fig. 1 Ishikawa et al.

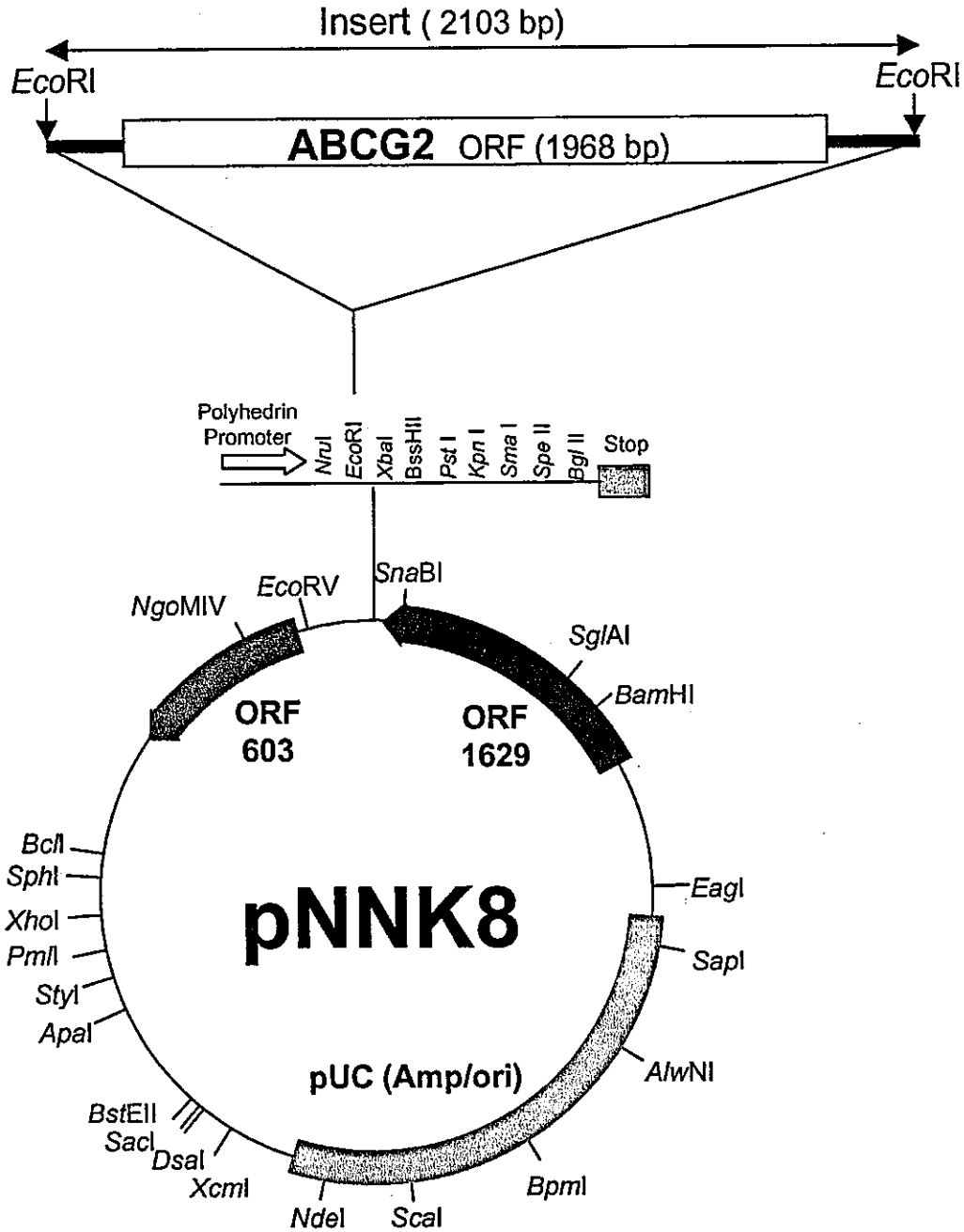


Fig. 2 Ishikawa et al.

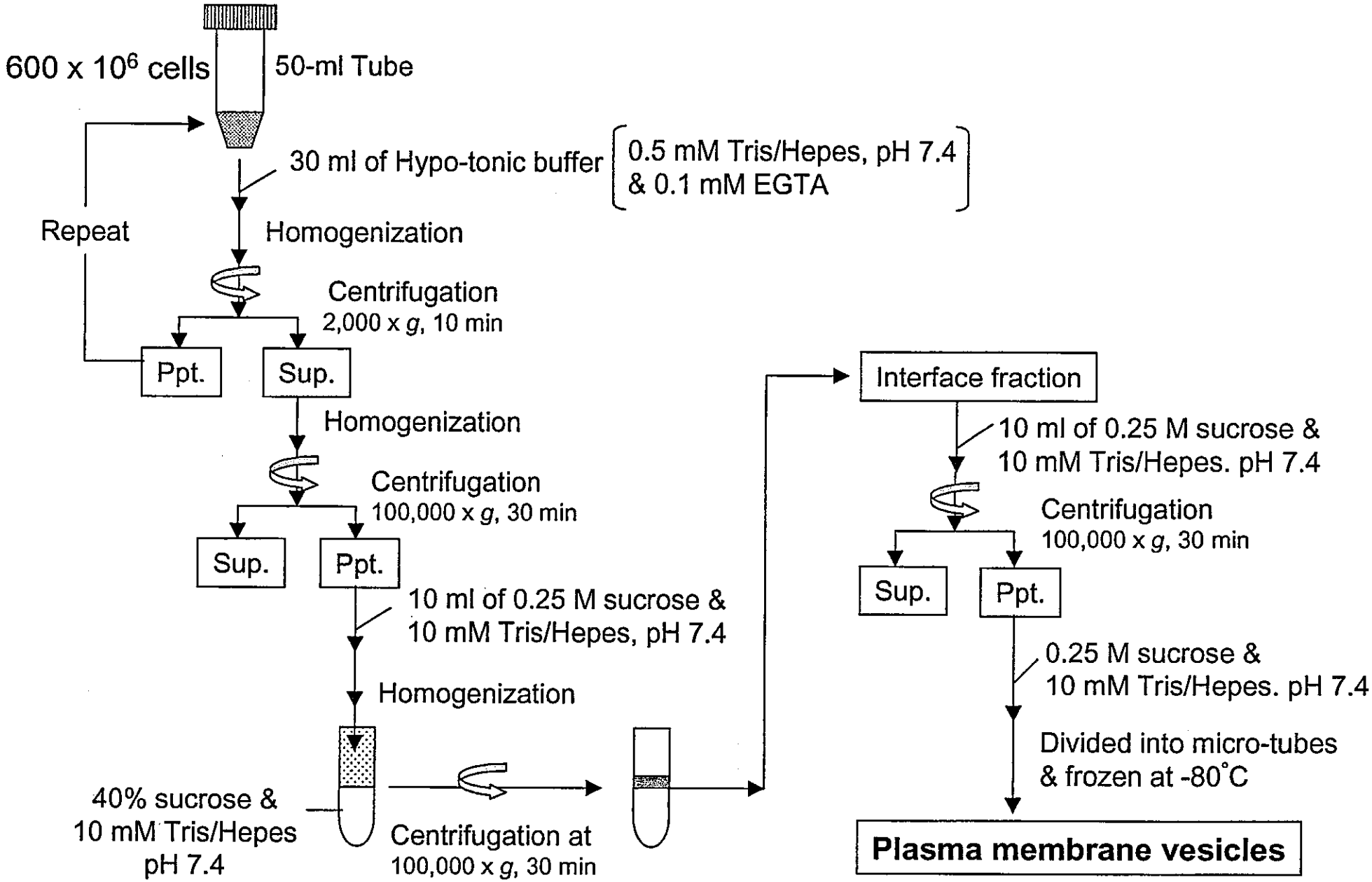


Fig. 3 Ishikawa et al.

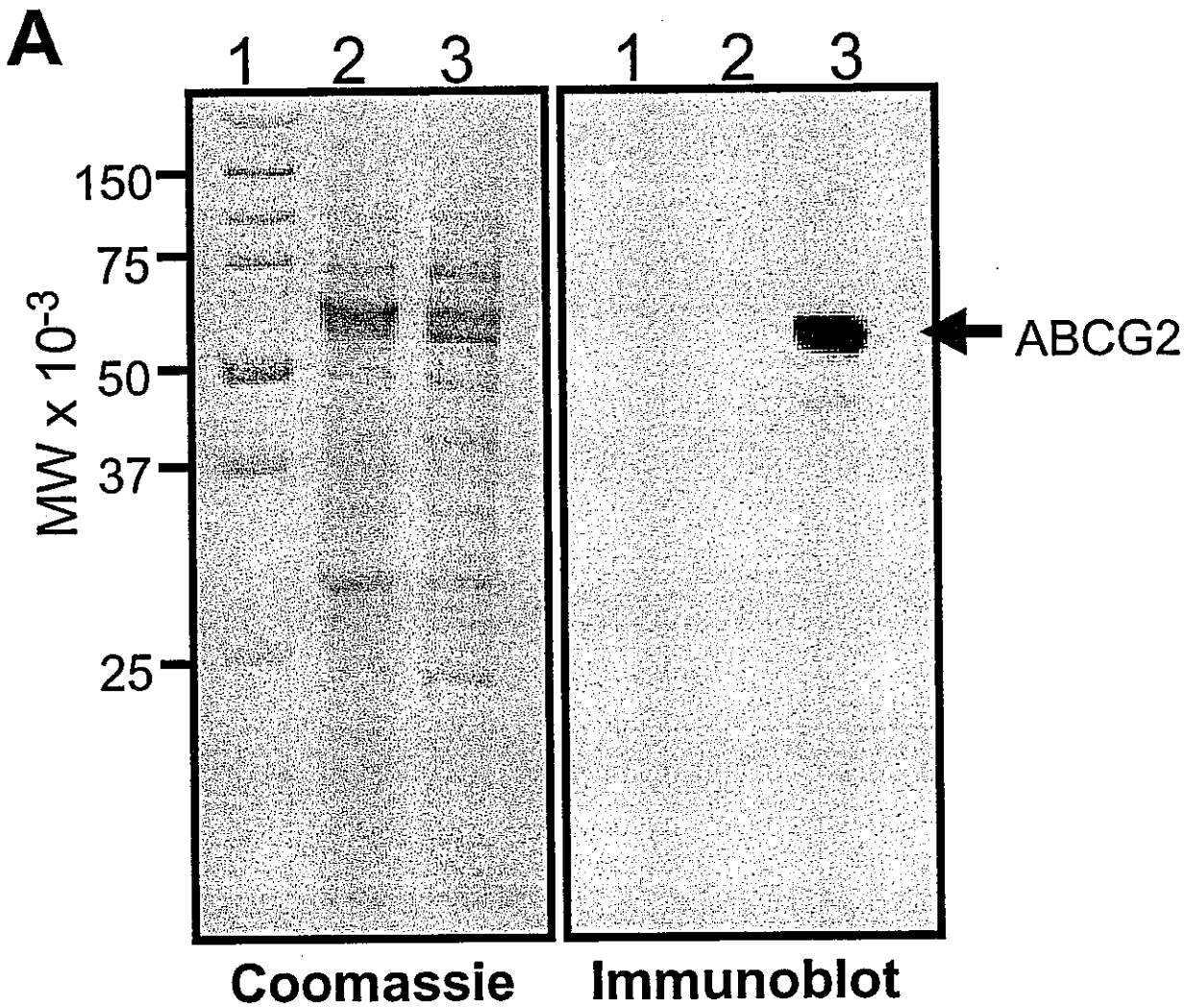


Fig. 4A Ishikawa et al.

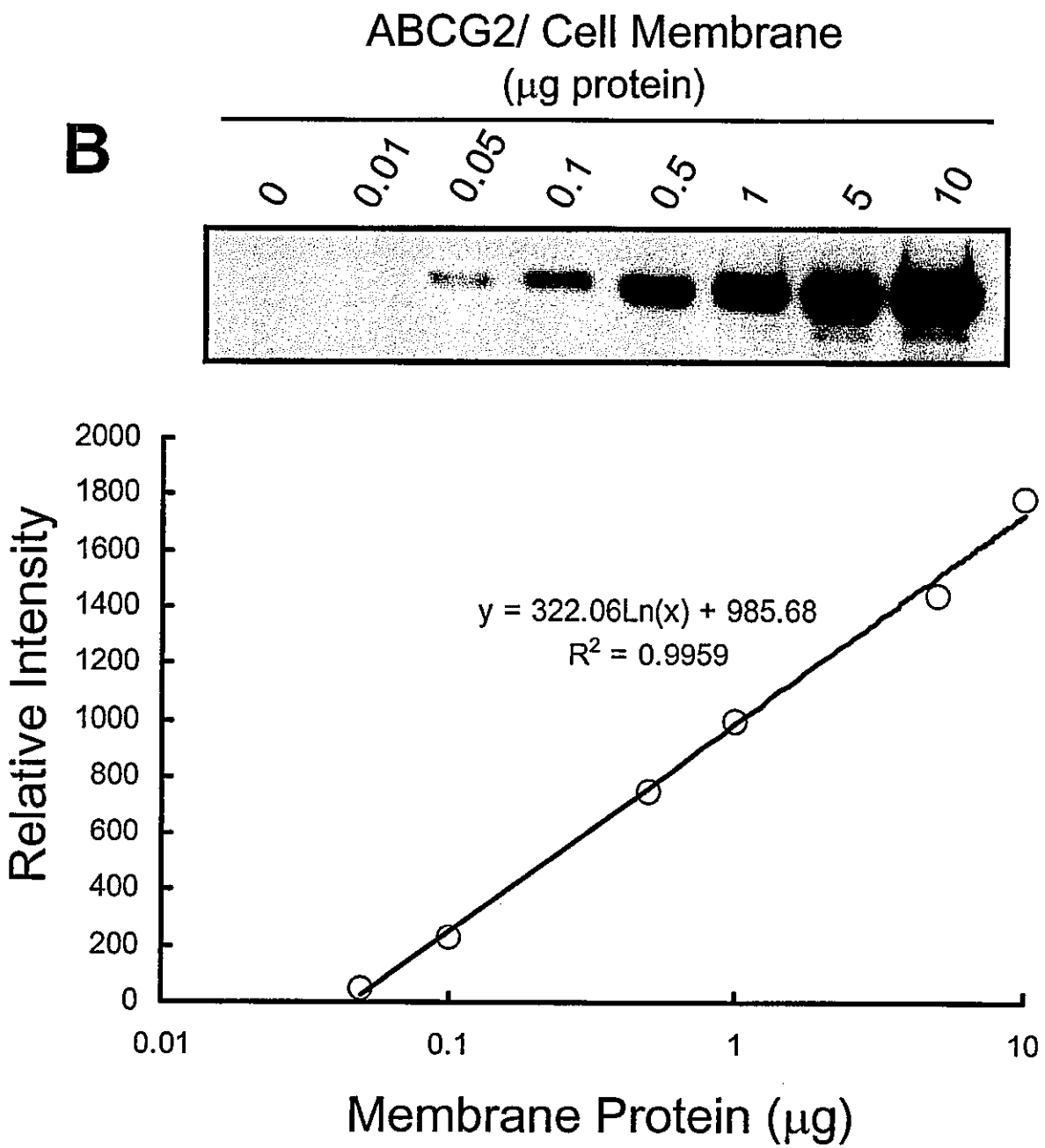


Fig. 4B Ishikawa et al.

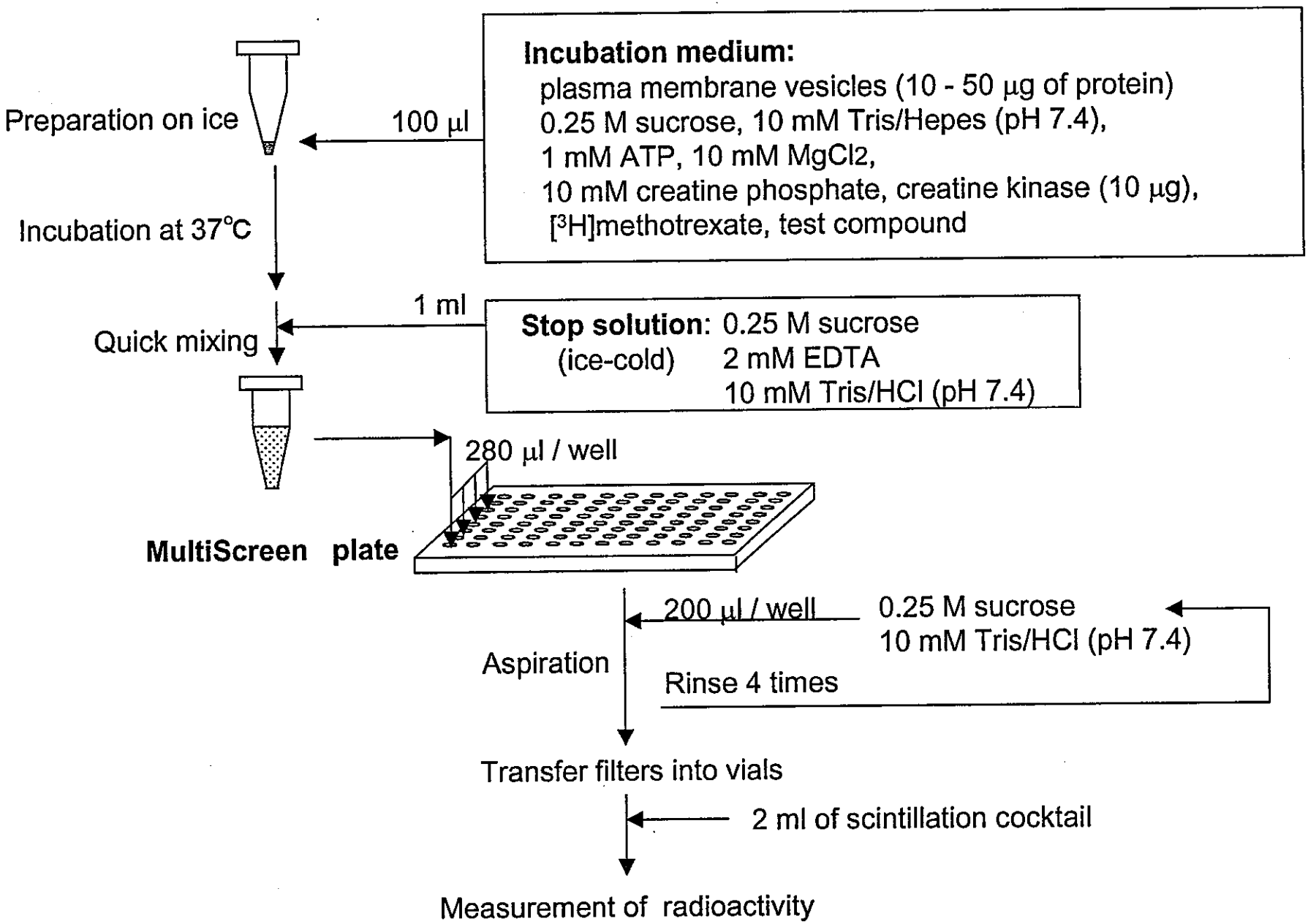
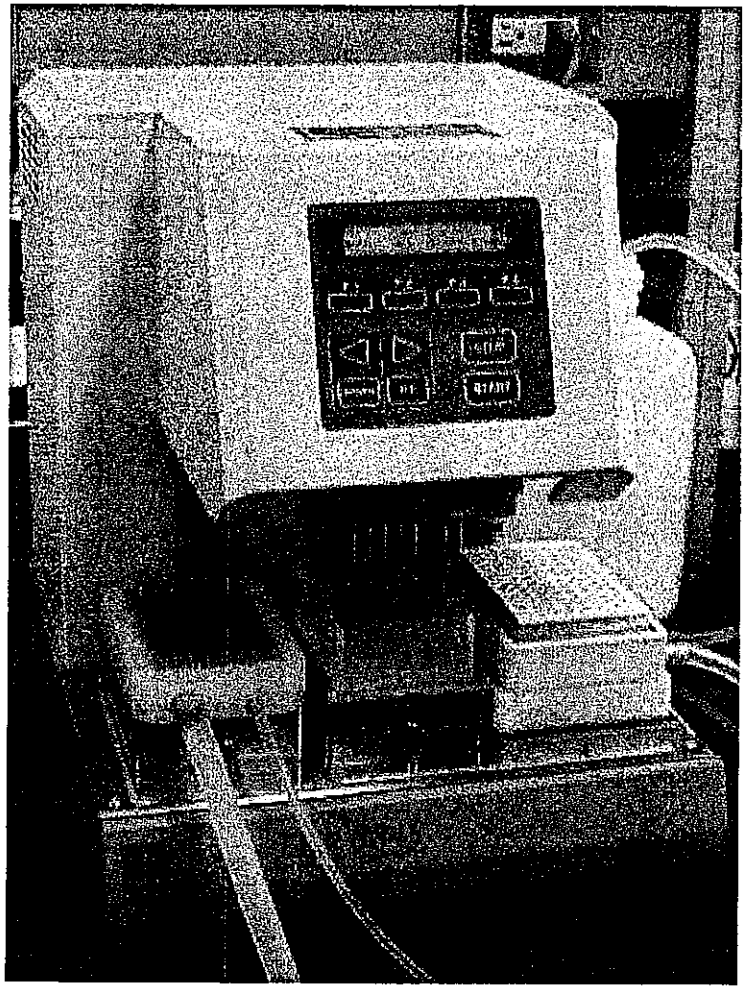


Fig. 5A Ishikawa et al.



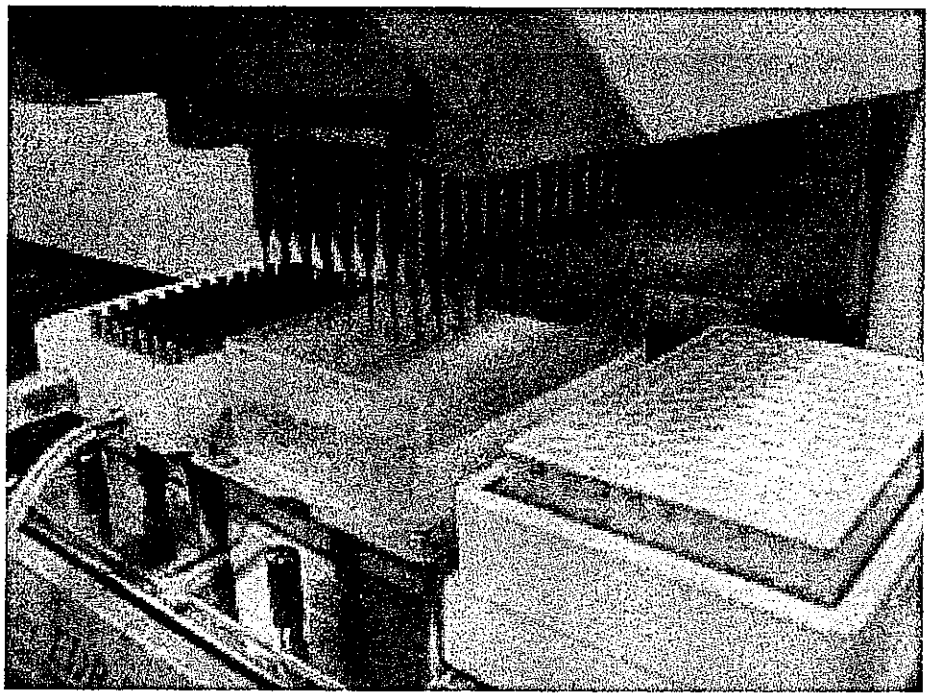


Fig. 5B Ishikawa et al.