Research Paper

Ex Vivo Induction of mRNA in Human Whole Blood as a New Platform of Drug and Dietary Supplement Development

Masato Mitsuhashi,1,2 Mieko Ogura,1 Katsuya Endo,2 Kazuhiko Obara,2 Hiroshi Izutsu,2 Stephan R. Targan,3 Motoko Maemura,4 Daisuke Tachikawa,4 and Atsushi Shinagawa5

Received July 16, 2007; accepted November 21, 2007; published online January 9, 2008

Purpose. We introduced a new concept of ex vivo gene expression analysis (Mitsuhashi, Clin Chem 53:148–149, 2007), where drug action was simulated under physiological conditions. This model system was applied to study various fields of drug development.

Materials and Methods. Heparinized human whole blood was incubated with drugs for less than 4h. The changes of specific mRNA were then quantified using the method we developed (Mitsuhashi, Tomozawa, Endo, and Shinagawa, Clin Chem 52:634–642, 2006).

Results. The mRNA quantitation method was used as a model system to study the following areas: (1) identification of respondents and non-respondents, (2) ex vivo compound screening, (3) determination of individually optimized doses, (4) drug-to-drug comparison, (5) assessment of leukocyte toxicity, (6) discovery of molecular targets, (7) assessment of the action of dietary supplements, and (8) characterization of respondents and non-respondents for various dietary supplements.

Conclusion. Since ex vivo assays are safe, a large number of healthy donors and disease patients can be recruited to identify individual-to-individual variations, which is not available from current preclinical study models. Although each system should be validated using a large number of samples, the ex vivo analysis will be a new tool for the development of drugs and dietary supplements in future.

KEY WORDS: blood; dietary supplement; drug development; gene expression; mRNA.

INTRODUCTION

Drug candidates are usually selected by various cell-free systems followed by a series of experiments with cultured cells and experimental animals. The final one then moves to clinical trials. However, because of the gap between these preclinical study models and the human body, many drug candidates fail during clinical trials. Some drugs are very effective in cultured cells or animal models, but fail to show any efficacy in humans. Moreover, unexpected severe side effects may happen only in human. Since the clinical trials are extremely expensive and have a potential for life-threatening adverse reactions, the improvement in preclinical studies is clearly one of the main priorities of pharmaceutical research.

Although early stage drug candidates cannot be administered to humans, it is technically and ethically feasible to mix these drugs in human whole blood to evaluate the efficacy and toxicity. It is particularly reasonable if such drugs are planned to be administered intravenously or expected blood levels are predetermined by mathematical models or animal experiments. Because in vitro assays are safe, a large number of healthy and disease donors can be recruited. Unfortunately, such blood experiments do not analyze drug actions on organs or whole body. However, it is at least useful to identify the effect on leukocytes. Thus, the methodology is primarily applicable to anti-inflammatory and immunomodulatory drugs. It may be useful for other drugs to assess adverse effects on leukocytes, because leukocyte toxicity is occasionally fatal. Moreover, leukocytes may be used as a surrogate marker for various diseases.

Typically, mononuclear leukocytes are isolated from human whole blood, suspended in culture media, incubated in a CO2 incubator for a couple of days to several days with drugs, and various drug-related protein syntheses and biological functions are determined. However, the assay condition is no longer physiological due to a lack of communication among different cell populations, oxygen supply from erythrocytes, as well as complex interactions with plasma proteins and other components. Secondary reactions may happen during the lengthy
incubation period. In order to avoid these problems, our group recently reported a novel assay system, in which human whole blood was stimulated with appropriate drugs *in vitro* for less than 4h, and drug-induced mRNA expression was quantified (1,2). The use of whole blood and a short incubation period are very physiological and considered as ex vivo.

Although the induction of mRNA does not always correspond to protein synthesis and subsequent biological and clinical outcomes due to altered splicing, post-translational modification, co-activation of inhibitory cascades, etc., the induction of appropriate mRNA under *ex vivo* condition in each patient will be an interesting research model for drug development. Especially, this *ex vivo* assay may become a powerful tool for the analysis of individual-to-individual variations, which is a foundation of tailored medicine, and is difficult to assess by current preclinical studies. In order to emphasize the value of *ex vivo* assay, various applications are demonstrated in this study, although each system should be validated clinically using appropriate drug targets.

### MATERIALS AND METHODS

#### Materials

Anti-T cell receptor (TCR) monoclonal antibody (IgG1 κ), control mouse IgG1 κ (BioLegend, San Diego, CA, USA), GSK-3β inhibitor I, JNK inhibitor II, JAK inhibitor I, Aloisine A, (Calbiochem, San Diego, CA, USA), fucoidan (Dr. Fucoidan, Fukuoka, Japan), and Hokkaido System Science (Sapporo, Japan). Oligo Service (Tsukuba, Japan), Nippon EGT (Toyama, Japan), Mediage Shirogane (Fukuoka, Japan) after respective Institutional Review Board (IRB) approvals. Blood samples were stored at 4°C, and stimulated with appropriate drugs in the same day of blood draw. After each drug treatment, blood samples were stored frozen at −80°C.

#### Blood Sampling

Heparinized whole blood samples were obtained from Apex Research Institute (Tustin, CA, USA), Hitachi, Ltd., Hitachi General Hospital (Hitachi, Japan), Cedars-Sinai Medical Center (Los Angeles, CA, USA), and Salon de Mediage Shirogane (Fukuoka, Japan) after respective Institutional Review Board (IRB) approvals. Blood samples were stored at 4°C, and stimulated with appropriate drugs in the same day of blood draw. After each drug treatment, blood samples were stored frozen at −80°C.

#### Primers and Probes

Polymerase chain reaction (PCR) primers and TaqMan probes were designed by Primer Express (Applied Biosystem, Foster City, CA, USA) and HYBSimulator (RNAture, Irvine, CA, USA) (3,4) (Supplemental Table 1). Oligonucleotides were synthesized by IDT (Coralville, IA, USA), Tsukuba Oligo Service (Tsukuba, Japan), Nippon EGT (Toyama, Japan), and Hokkaido System Science (Sapporo, Japan).

#### Blood Stimulation

In eight-well strip microtubes, 1.2–1.4μl of 50x concentrations of drugs or controls [phosphate buffered saline (PBS) or dimethylsulfoxide (DMSO)] were added, and stored at −20°C until use. About 60–70μl of blood samples was added into each well in triplicate, and incubated at 37°C for 2–4h with cap closed. In some experiments (Figs. 2, 3 and 8), inhibitors were added 30min–1h prior to the stimulations. After each treatment, 50μl of whole blood was transferred to filterplates as described in the next section.

#### mRNA Quantification

The quantity of mRNA was determined by the method we developed previously (1,2). In brief, 50μl of blood samples were applied to 96-well filterplates to trap leukocytes. Sixty microliters of lysis buffer containing a cocktail of specific reverse primers was added to the filterplates, and the resultant cell lysates were transferred to oligo(dT)-immobilized microplates (GenePlate, RNAture) (5,6) for poly(A)+ mRNA purification. The cDNA was directly synthesized in 30μl solutions at each well: specific primer-primed cDNA in the liquid phase and oligo(dT)-primed cDNA in the solid phase (1). Four microliters of undiluted (Figs. 1, 2, 3, 4, 5, 7 and 8) or diluted (Figs. 6) cDNA solution was used for TaqMan PCR (Figs. 3, 4, 5 and 8) or SYBR Green PCR (Figs. 1, 2, 6 and 7) using iTaqSYBR master mix (BioRad, Hercules, CA, USA), as described previously (1,2). Each gene was amplified individually. The cycle threshold (Ct), which was the cycle of PCR to generate certain amounts of PCR products (fluorescense), was determined using analytical software (SDS, Applied Biosystems). In order to maintain the assay specificity of SYBR Green PCR, 1× RT buffer was used as negative controls to confirm no primer dimer was generated under this PCR condition. Moreover, the
melting curve was analyzed in each case to confirm that PCR signals were derived from a single PCR product. We also measured standard curves during PCR using known concentrations (1–10⁶ copies/well) of synthetic DNA templates, and found that the efficiency of PCR was always close to 100% under this PCR condition (annealing for 30s plus extension for 1min) regardless of target species, because tenfold differences of the amounts of templates were approximately 3.3 in ΔCt (2^3.3 = 9.8 folds). More importantly, the amplification slope of the samples was always identical to that of corresponding templates, suggesting that PCR efficiency was conserved between the templates and samples. Thus, the Ct values of drug-treated triplicate samples were subtracted by the mean Ct values of control samples to calculate ΔCt, and the fold increase was calculated as 2^(−ΔCt).

RESULTS AND DISCUSSION

Identification of Respondents and Non-Respondents

Prediction of respondents and non-respondents against specific drug treatment is critically important for clinical trials. Once non-respondents are identified before drug treatment, such patients will be excluded from the clinical trial to improve the chance of success of the study. Figure 1 was an initial attempt to this field. Mouse monoclonal antibody IgG1κ against human α/β T cell receptor (TCR) induced tumor necrosis factor α (TNFα) mRNA in a dose dependent manner, when antibody was incubated in heparinized human whole blood for only 2h (Fig.1A, filled circle). Control mouse IgG1κ also increased TNFα mRNA (Fig. 1A, open circle), although the values were much smaller than those of anti-TCR. Thus, fold increase was calculated by using the same concentrations of control mouse IgG (Fig.1B). TCR activation was known to produce TNFα (9), however, we found that approximately one third of healthy population increased

![Fig. 2. Ex vivo compound screening. Using three donors (1–3), 50 μl each of heparinized human whole blood was exposed to various drugs (all 10 μM in the final concentrations) for 1 h in triplicate, then stimulated with 10 μg/ml each of anti-TCR (filled circle) or control IgG (open circle) for an additional 2 h. TNFα (A) and CXCL10 (B) mRNA were quantified as described above. The y-axis was the Ct, the cycle of PCR to generate certain amounts of PCR products: the smaller Ct values mean higher expression, and 1 Ct difference means double or one half in quantity. Each data point is the mean±SD from triplicate aliquots of whole blood.](image)

![Fig. 3. PHA-induced IL-2 mRNA expression in human whole blood. A Blood samples were stimulated with various concentrations of phytohemagglutinin-P (PHA) or control (PBS) for 1 h, then IL-2 (filled circle) and β-actin (filled triangle) mRNA were quantified using TaqMan as described in the Methods. B Blood samples were pretreated with 50–400 nM of CsA (filled circle) or 5–40 nM of FK506 (open triangle) for 1 h, then stimulated with 30 μg/ml of PHA or control PBS at 37°C for another 1 h. The %Control was calculated by using the values of PBS (no PHA) and PHA (no inhibitor) as 0 and 100%, respectively, for each individual. C The IC50 of both CsA (y-axis) and FK506 (x-axis) was plotted. Each data point is the mean±SD (A) or mean (B, C) from each individual. The regression line, r², and p value are shown in the subpanel C.](image)
TNFα mRNA, whereas remaining two thirds failed to respond, even the highest concentrations of antibodies (10 μg/ml) were used (Fig. 1B). The y-axis of Fig. 1A and B is different, because the control values were solvent (PBS) (A) and control IgG at each time point (B), respectively. This is the first report showing ex vivo respondents and non-respondents for TCR-mediated TNFα mRNA induction, although its clinical implication is not yet characterized.

TCR is a heterodimer molecule of immunoglobulin-like α and β chains, and is associated with 4 molecules of CD3 (γ, δ, and 2ζ), 2 ζ chains, and CD8 (homo- or heterodimer of α and β chains) to form the TCR-CD3 complex. TCR α/β chains are the molecules to recognize the specific structure of the target to elicit various immunological reactions. Figure 1 was a model system to assess TCR functionality by activating TCR α/β chains with agonistic anti-TCR antibody. The components of TCR-CD3 complex have multiple transcript variants as well as various genetic polymorphisms (10–13). TCR-CD3 complex is also known to activate various kinases (14,15), and these kinases have multiple transcript variants and genetic polymorphisms, too (16). Thus, altered functions of TCR shown in Fig. 1 may happen by genetic alterations of any of these genes. Alternatively, non-respondents may have some inhibitor in plasma. However, it is time consuming, labor-intensive, and expensive to identify genetic polymorphisms of these genes as well as discovery of unknown plasma factors in each human subject. The identification of functional respondents and non-respondents under physiological ex vivo condition may be useful to classify the patient population for clinical trials of immunomodulatory drugs and vaccines, where TCR plays a crucial biological function.

**Ex Vivo Compound Screening**

Drug candidate compounds are usually screened by cell-free system or cultured cells. Figure 2 is a new concept of ex vivo screening directly using human whole blood. Each blood sample was exposed to various drugs 1h prior to anti-TCR stimulation, then TNFα (Fig. 2A) mRNA was quantified as described above. Furthermore, additional CXCL10 cytokine...
mRNA was also quantified (Fig. 2B). Blood volume used for each volunteer was less than 3 ml [8 (7 drugs and 1 control) × 2 (control IgG and anti-TCR) × 3 (triplicate) × 60 μl]. The y-axis was the Ct, the cycle of PCR to generate certain amounts of PCR products: the smaller Ct values mean higher expression, and 1 Ct difference means double or one half in quantity. In order to screen a wide spectrum of compounds, each donor was treated with different drugs. Three donors (all respondents) showed different expression levels of TNFα and CXCL10 mRNA (Fig. 2A, filled circle), and cilostazol (Fig. 2A) and rolipram (Fig. 2A,B) decreased the basal expression levels of these mRNA (Fig. 2, open circle). Anti-TCR antibody induced TNFα and CXCL10 mRNA (Fig. 2, filled circle), with the Ct difference approximately 2 (=4 fold increase) and 4–6 (=16–64 fold increase) for TNFα and CXCL10 mRNA, respectively, in these 3 donors. However, cyclosporine (CsA) and tacrolimus (FK506) completely eliminated the induction of TNFα mRNA (Fig. 2A, filled circle), whereas control β-actin mRNA was unchanged (Fig. 3A, filled circle). Pretreatment with cyclosporine (CsA) and tacrolimus (FK506) inhibited PHA-induced IL2 mRNA expression in a dose dependent manner (Fig. 3B), with an IC_{50} of 100–450 nM and 1–18 nM for CsA and FK, respectively (Fig. 3C). The results were similar to those reported by other groups (20,21), however, our method was more practical in terms of blood volume, throughput, and reproducibility.

The range of IC_{50} varies 5–20 folds, suggesting that drug doses may be determined individually by assessing IC_{50} for each patient. The practice of therapeutic drug monitoring

### Determination of Individually Optimized Doses

Unlike Fig. 1, many drugs show dose dependent reactions, and study subjects will be classified into low- and high-responders. For low respondents, drug doses should be increased to induce appropriate clinical responses, whereas drug doses should be decreased for high-respondents to eliminate unwanted side effects. Determination of individually optimized doses is not only useful for clinical practice as a tailored medicine, but also applicable to study design of clinical trials in future. Figure 3 demonstrated an example of such dose-dependent ex vivo analysis. Phytohemagglutinin-P (PHA) increased IL2 mRNA in a dose dependent manner (Fig. 3A, filled circle), whereas control β-actin mRNA was unchanged (Fig. 3A, filled triangle). Pretreatment with cyclosporine (CsA) and tacrolimus (FK506) inhibited PHA-induced IL2 mRNA expression in a dose dependent manner (Fig. 3B), with an IC_{50} of 100–450 nM and 1–18 nM for CsA and FK, respectively (Fig. 3C). The results were similar to those reported by other groups (20,21), however, our method was more practical in terms of blood volume, throughput, and reproducibility.

![Fig. 5. Bleomycin (BLM)-induced p21 and PUMA mRNA expression in human whole blood. Blood samples from 18 healthy adult volunteers were stimulated with 0–10 μM BLM for 4 h, then p21 (A) and PUMA (B) mRNA were quantified using TaqMan as described in the “Materials and Methods”. Each data point is the mean from triplicate whole blood samples in each individual. Hashed areas are considered as negative response with <1.5 fold increase.](image)

![Fig. 6. Screening of tumor necrosis factor superfamily (TNFSF) mRNA. Blood samples were stimulated with either 100 μg/ml each of PHA (A, n=12) or concanavalin A (B, n=3), 10 μg/ml lipopolysaccharide (LPS) (C, n=4), or 200 μg/ml of heat aggregated human IgG (HAG) (D, n=20) at 37°C for 4 h, then all members of TNFSF mRNA were quantified using SYBRgreen PCR and fold increase was calculated by using the values of solvent (PBS) as control, as described in the Methods. The cDNA was diluted 1:4 with water, and used for PCR. TNFSF10, 11, 18 showed some primer dimers. Each data point is the mean from triplicate whole blood samples in each individual.](image)
(TDM) was introduced many years ago when new technologies allowed us to determine the levels of drug concentrations in serum or plasma. Functional variations determined by the ex vivo analysis will be a new addition to TDM in future.

Although high respondents to CsA were also high respondents to FK506, variation existed among individuals (Fig. 3C). This may indicate the possibility of drug preference between CsA and FK506. This is reasonable because CsA and FK506 are acting against different molecular targets [cyclophilin for CsA (22) and FK506 binding protein for FK506 (23)], although both inhibit calcineurin.

Drug-to-Drug Comparison

When a new drug is introduced to the market, it is required to show advantages of such drug against existing ones, if similar drugs are available. Using whole blood samples from leukemia patients, where more than 96%...
Assessment of Leukocyte Toxicity

Although leukocyte toxicity is not a major problem for anti-leukemia drugs, it becomes a huge problem for anticancer drugs for solid tumors, because it sometimes becomes fatal. Unfortunately, this side effect is unpredictable. Figure 5 is an example, where 0–10μM bleomycin (BLM) were used to stimulate whole blood and p21 and PUMA mRNA were quantified under the identical condition to that of Fig. 4. As shown in Fig. 5, the responses were dose dependent for both p21 and PUMA, and all individuals showed more than 1.9 fold increase at 10μM BLM. Interestingly, approximately half [56% (A) and 44% (B)] of healthy individuals responded to 0.1μM BLM to induce p21 and PUMA mRNA, respectively, whereas the other half did not. Among them, one individual failed to induce both p21 and PUMA mRNA even at 1μM of BLM. The results of Fig. 5 do not directly indicate the predictability of leukocyte toxicity, because toxic BLM is not allowed to be administered to healthy individuals. However, the existence of toxicity in patients may provide additional considerations for the development of chemotherapeutic drugs.

Discovery of Molecular Targets

In Fig. 3, PHA-induced IL-2 mRNA was quantified, because the system was previously reported (20,21). Then, the remaining cDNA was further used to screen various mRNA. Figure 6 illustrates the results of the screening of tumor necrosis factor superfamily (TNFSF) mRNA. As shown in Fig. 6A, PHA induced TNFSF2 (=TNFα), 5 (=CD40 ligand), 6 (=Fas ligand), 9, and 14 (=LIGHT, CD258). B-cell stimulator lectin, concanavalin A (ConA) induced TNFSF2, 6, 9, and 14 mRNA, but not TNFSF5 mRNA (Fig. 6B). Lipopolysaccharide (LPS), which is known to activate Toll-like receptor-2 (28) and 4 (29) induced mainly TNFSF2 mRNA (Fig. 6C). Moreover, the classic model of immune complex, heat aggregated IgG (HAG) (30) induced different classes of TNFSF, such as TNFSF8 (CD30 ligand, CD153) and 15 (=TL1A) as well as TNFSF2, 9 and 18 in some cases (Fig. 6D). Since human genome and mRNA sequences are available, new molecular targets can be discovered by ex vivo screening as shown in Fig. 6.

HAG-induced TNFSF mRNA may be an interesting clinical research model of antibody medicines, because therapeutic antibodies will form immune complexes in the body by binding to the target molecules. The immune complexes are known to activate IgG Fc receptors (FcγR), and FcγR-mediated functions are consisted of numerous variants, such as 4 subclasses of Fc portions of IgG (IgG1–4), multiple classes of FcγR [FcγRIa, b, c (=CD64), FcγRIIa, b, c (=CD32), and FcγRIIIa and b (=CD16)], different subsets of FcγR-bearing leukocytes, and various inducible cytokines. Moreover, these proteins have multiple transcript variants as well as various genetic polymorphisms (31,32). As shown in Fig. 6D, HAG showed respondents and non-respondents for each of TNFSF mRNA, and these variations may happen at any of these genes. The individual variations of ex vivo responses shown in Fig. 6D may be a predictable marker for the efficacy and toxicity of antibody medicines.

Assessment of Acute Action of Dietary Supplements

Although various dietary supplements are used widely, the scientific evidence for their efficacy is not well established. One of problems of dietary supplement research is the lack of scientific and quantitative methods to assess acute actions, because chronic effects are difficult to interpret. Since dietary supplements are freely available, we can analyze the effects of dietary supplements before and after ingestion for each individual. Figure 7 was an example of fucoidan, the sea weed extract used widely in Japan. We collected blood samples before and 4 hours after ingestion of 50ml of fucoidan (2,550mg), and analyzed the changes of HAG-induced TNFSF15 and IL8 mRNA induction. As shown in Fig. 7A, HAG (100μg/ml) induced TNFSF15 and IL8 in a dose dependent manner. Although the levels of HAG-induced TNFSF15 mRNA expression were unchanged between these 2 samples (Fig. 7B), the levels of HAG-induced IL8 mRNA was significantly reduced after fucoidan ingestion (Fig. 7C). The same individuals were tested two to four times (Fig. 7C, each symbol represents the same individual), however, the effects of fucoidan was confirmed every time. Although the biological and clinical meanings of the results are not known at this time, it is surprising to find such significant effects within 4h of ingestion. The ex vivo assay will be sensitive enough to analyze the efficacy of dietary supplements in each individual.

Characterization of Respondents and Non-respondents for Dietary Supplements

Dietary supplements, such as vitamins, polyphenols, turmeric, etc. are known to induce various biological activities in cultured cells and animals, and some of activities were confirmed by subsequent clinical studies. However, the results of clinical studies are difficult to interpret because of the inconsistency of the results. If respondents and non-respondents...
Ex Vivo Platform of Drug Development

exist, and the later population is much larger than the former, the standard method of double blind clinical trials is no longer useful. Since the blood levels of active ingredients of dietary supplements were well characterized, simulation will be theoretically possible by incubating whole blood with pure ingredients at designated blood levels. In Fig. 8, human whole blood was pretreated with various ingredients 1 hour prior to PHA-induced IL2 mRNA expression analysis. The concentrations of these ingredients used in Fig. 8 (0.1 μM vitamin A, 10 μg/ml vitamin C, 10 μM epigallocatechin gallate (green tea), 2 μM genistein (soybean), and 200 nM curcumin (turmeric) were achievable blood levels according to various literature. The effects of dietary supplements were much weaker than active drugs as shown in Figs. 2 and 3, however, some showed significant influences on the basal expression levels of IL2 mRNA as well as PHA-induced IL2 mRNA expression (Fig. 8, filled circle, filled triangle). However, such effects were not identified for all individuals. For example, vitamin A enhanced PHA-induced IL2 mRNA expression in four (Donor 1, 2, 5, 7) out of seven individuals, whereas vitamin C inhibited the reaction in three cases (Donor 3, 4, 6) (Fig. 8B, filled triangle). The ex vivo simulation was sensitive enough to identify respondents and non-respondents for dietary supplements, although biological and clinical meanings are not clear at this time. However, identification of individual variation will be a useful tool for the clinical studies of diet and dietary supplements in future.

CONCLUSIONS

The ex vivo analysis is a new concept we introduced recently by using an mRNA quantification platform we developed (1,2). In order to explore the possibility of ex vivo assay, various applications are demonstrated in this study. Although mRNA expression does not always correspond to protein synthesis and subsequent biological and clinical outcomes, identification of appropriate mRNA induction in each individual will be a useful information for the development of drugs and dietary supplements. Since 450 ml of blood can be obtained from a single individual (450 ml is a unit of blood donation for transfusion in the US), and the ex vivo assay uses as little as 50 μl per reaction (150 μl in triplicate for each drug), as many as 3,000 drug compounds can be screened. Thus, we believe that the proposed ex vivo assay will be a new addition to pharmaceutical and dietary supplement research.

ACKNOWLEDGMENTS

We would like to thank Y. Oka, S. Nemoto, N. Sasaki and staffs of clinical laboratories (Hitachi General Hospital, Hitachi, Japan), Dr. MJ Tonkon, C. Fox, C. McGinty, and J. Haire (Apex Research Institute, Tustin, CA, USA), C. Landers and J. Price (Cedars Sinai Medical Center, Los Angeles, CA, USA), K. Nakamizo (Ventuno, Fukuoka, Japan), K. Otsuka (Kamerica, Torrance, CA, USA), T. Otake, H. Ito, and B. Maekawa (Hitachi Chemical, Tokyo, Japan) for the support of the blood draw and clinical section of this study. We also thank C. Yamamoto (Hitachi Chemical Research Center, Irvine, CA, USA) and I. Perlinski (Perlinski and Company, Laguna Niguel, CA, USA) for the review of English.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution and reproduction in any medium, provided the original author(s) and source are credited.

REFERENCES

1. M. Mitsuhashi, S. Tomozawa, K. Endo, and A. Shinagawa. Quantification of mRNA in whole blood by assessing recovery of RNA and efficiency of cDNA synthesis. Clinc Chem 52:634–642 (2006).
2. M. Mitsuhashi. Ex vivo simulation of drug action: Quantification of drug-induced mRNA as a bridge between preclinical and clinical trials. Clin. Chem 53:148–149 (2007).
3. M. Mitsuhashi, et al. Oligonucleotide probe design—a new approach. Nature 367:759–761 (1994).
4. D. Hyndman, et al. Software to determine optimal oligonucleotide sequences based on hybridization simulation data. BioTechniques 20:1090–1097 (1996).
5. M. Mitsuhashi, C. Keller, and T. Akitaya. Gene manipulation on plastic plates. Nature 357:519–520 (1992).
6. Y. Hamaguchi, et al. Direct reverse transcription-PCR on oligo (dT)-immobilized polypropylene microplates after capturing total mRNA from crude cell lysates. Clin. Chem 44:2256–2263 (1998).
7. P. M. Holland, et al. Detection of specific polymerses chain reaction product by utilizing the S-3′ exomicnuclease activity of Thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. U.S.A 88:7276–7280 (1991).
8. T. B. Morrison, J. J. Weis, and C. T. Wittwer. Quantification of low-copy transcripts by continuos SYBR Green I monitoring during amplification. Biotechniques 24:954–962 (1998).
9. I. Millet, and N. H. Ruddle. Differential regulation of lympho- toxin (LT), lympho-toxin-beta (LT-beta), and TNF-alpha in murine T cell clones activated through the TCR. J. Immunol 152:4336–4346 (1994).
10. P. Charmlay, et al. Human T-cell receptor C3d-epsilon (C3dE)/ TaelI DNA polymorphism. Nucleic Acids Res 17:2374(1989).
11. U. Malhotra, and P Concannon. Human T-cell receptor C3d- delta (C3dD)/MspI DNA polymorphism. Nucleic Acids Res 17:2373(1989).
12. P. Charmlay, et al. Human T-cell receptor C3d-gamma (C3dG)/ MspI DNA polymorphism. Nucleic Acids Res 17:2372 (1989).
13. M. H. Polymero, R. H. Xia, D. S. Rath, and C. R. Meuer. Dinucleotide repeat polymorphism at the human MHC class I CD8 alpha-chain (Leu-2/T8) gene. Nucleic Acids Res 19:1718 (1991).
14. S. Gibson, et al. The EMT/ITK/TSK (EMT) tyrosine kinase is activated during TCR signaling: LCK is required for optimal activation of EMT. J. Immunol 156:2716–2722 (1996).
15. J. A. Fearson, and D. R. Alexander. The phosphotyrosine phosphatase SHP-2 participates in a multimeric signaling complex and regulates T cell receptor (TCR) coupling to the Ras/ mitogen-activated protein kinase (MAPK) pathway in Jurkat T cells. J. Exp. Med 187:1417–1426 (1998).
16. S. Nervi, et al. No association between lck gene polymorphisms and protein level in type 1 diabetes. Diabetes 51:3320–3323 (2002).
17. T. Espevik, et al. Inhibition of cytokine production by cyclosporin A and transforming growth factor beta. J. Exp. Med 166:571–576 (1987).
18. A. D. Cristillo, M. J. Macir, and B. E. Bierer. Differential chemokine expression profiles in human peripheral blood T lymphocytes: dependence on T-cell coreceptor and calcium signaling. Blood 101:216–25 (2002).
19. N. Kanda, and S. Watanabe. Prolactin enhances interferon-gamma-induced production of CXC ligand 9 (CXCL9), CXCL10, and CXCL11 in human keratinocytes. Endocrinology 148:2317–2325 (2007).
20. T. Giese, et al. Monitoring of NFAT-regulated gene expression in the peripheral blood of alograft recipients: a novel perspective
toward individually optimized drug doses of cyclosporine A. Transplantation 77:339–344 (2004).

21. C. Hartel, et al. Sensitivity of whole-blood T lymphocytes in individual patients to tacrolimus (FK 506): impact of interleukin-2 mRNA expression as surrogate measure of immunosuppressive effect. Clin. Chem 50:141–151 (2004).

22. R. E. Handschumacher, M. W. Harding, J. Rice, R. J. Druge, and D.W. Speicher. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 226:544–547 (1984).

23. J. J. Siekierka, S. H. Hung, M. Poe, C. S. Lin, and N. H. Sigal. A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature 341:755–757 (1989).

24. J. Pines. Cell cycle. p21 inhibits cyclin shock. Nature 369:520–521 (1994).

25. J. Han, et al. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc. Natl. Acad. Sci. U.S.A 98:11318–11323 (2001).

26. J. Yu, et al. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol. Cell 7:673–682 (2001).