Article title:

Quantitative RT-PCR measurement of human cytochrome P-450s. Application to drug induction studies.

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ABSTRACT

A quantitative RT-PCR assay has been developed to measure the mRNA contents of the major CYPs (1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5) in human liver samples as well as in cultured human hepatocytes. The technique is highly specific, rapid and sensitive enough to quantitate low abundant mRNAs. The PCR primers were selected to specifically match each CYP mRNA, to have a very close anneling temperature, and to render PCR products of similar sizes. The PCR conditions were designed to facilitate the simultaneous measurement of the various human liver CYPs in a single run.

To allow precise and reproducible quantitation of each cytochrome mRNA content, a external standard (luciferase mRNA) is added to the probes to monitore the efficiency of the RT step. Quantitation of the amplification process is made using appropriate cDNA standards and a fluorescent measurement of the amplified products.

This assay has been applied to quantify the most relevant CYPs in human liver and cultured human hepatocytes. CYPs 3A4 and 2E1 mRNAs were the most abundant in human liver (2.5 and 1.7 x 10^8 molecules /µg of total RNA respectively) whereas 1A1 and 2D6 were the least abundant isoforms (1.2 and 2.1 x 10^6 molecules /µg of total RNA). A similar pattern was also found in human short-term cultured hepatocytes.

This technique is also suitable for assessing CYP induction by xenobiotics. Cells exposed to 3-methylcholanthrene showed a characteristic increased expression of CYP1A2 and 1A1. Upon incubation with phenobarbital and rifampin human hepatocytes increased CYP 2B6, 3A4 and 3A5 between others.

Key Words: Cytochrome P-450; RT-PCR; human hepatocyte; quantitative; CYP induction.

INTRODUCTION

The cytochrome P-450 (CYP) is a family of heme containing monooxygenases that catalyzes the oxidative metabolism of many endogenous and exogenous compounds (1). These enzymes play a major role in the biotransformation of xenobiotics (2-4), with the far highest concentration found in the liver. (5-7).

Different approaches have been developed to measure CYP expression in biological samples. The most common ones are based on the determination of the enzymatic activity of microsomal fractions using CYP specific substrates (3, 8, 9), as well as western immunoblot analysis using anti CYP antibodies (10). Although these techniques are reliable, both have limitations. The lack of specific substrates/inhibitors for all CYPs, makes it difficult to estimate the abundance of all relevant isoforms. Regarding western analysis, quantitation relies on the affinity and specificity of each antibody making the technique only semi-quantitative.

Another possible approach is to determine the mRNA content of each CYP isoform. The RT-PCR is a potent method able to efficiently and selectively measure mRNA quantities which are undetectable by other techniques, however, quantitative measurements are not simple. One frequently used strategy is the competitive PCR assay (11), where increasing amounts of a DNA highly homologous to the target, but distinguishable by size or restriction sites, is added to the polymerase chain reaction. Thereafter, both PCR products, competitor and target DNAs, are quantified. An inherent problem to all PCR assays is that it has to be assumed that the amplification efficiency for both standard and target sequence will be exactly the same. Another source of inaccuracy is the lack of control over the RT step.

In this study we describe a sensitive RT-PCR analysis for the quantitation of the most relevant xenobiotic metabolizing human CYP, namely, 1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5. Using suitable standards, the efficiency of both the RT and PCR

steps are determined. This allows to estimate the number of molecules of each CYP mRNA per µg of total isolated RNA. This assay allows measuring high and low abundant mRNAs in both liver samples and cells. The assay has also shown its suitability for an easy monitoring of the CYP induction by xenobiotics in human hepatocytes.

MATERIALS AND METHODS

Chemicals and reagents. Phenobarbital, rifampin, and 3-methylcholanthrene were purchased from Sigma. Oligonucleotide primers were custom-synthesized by GIBCO-BRL. Oligo d(T)14 primer was from GIBCO-BRL. RNA/ DNA modifying enzymes were all purchased from GIBCO-BRL. Ribogreen and picogreen were from Molecular Probes, Rneasy Total RNA Kit was from Quiagen. High pure PCR product purification kit was purchased from Boehringer. Luciferase RNA was from Promega.

Cell culture. Surgical liver biopsies were obtained from patients undergoing programmed cholecystectomy after informed consent. Human hepatocytes were isolated using a two-step perfusion technique (12) and seeded on 6 cm-diameter fibronectin-coated plates (3.6 µg/cm^2) at a density of 8x10^4 cells/cm^2 in an appropriate volume of medium. Culture medium was Ham F-12/Leibovitz L-15 (1/1, v/v), supplemented with 2% newborn calf serum, 5mM glucose, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.2% bovine serum albumin and 10^{-8}M insulin. Medium was changed 1h later to remove unattached hepatocytes.

Treatment of cultures. 24 hours after plating, human hepatocytes were exposed to inducers for 48 hours. Phenobarbital was prepared as an aqueous solution and added directly to cultures to a final concentration of 1 mM. 3-methylcholanthrene and rifampin were dissolved in DMSO and added to culture medium to give a final concentration of 2 µM and 50 µM, respectively. DMSO concentration in culture medium never exceeded 0.1% (v/v).

Isolation and purification of total RNA from liver tissue and cell cultures. Total RNA

was extracted from approximately 50 mg of human liver, or from 6 cm diameter culture plates of human hepatocytes using “Rneasy Total RNA Kits”. The amount of purified RNA was estimated by ribogreen fluorescence and its purity by the absorbance ratio 260/280 nm. RNA was incubated for 15 min at 23ºC with DNase I (1 unit/µg) according to the recommendations of the supplier, followed by thermal inactivation of the enzyme (65ºC for 10 min) in the presence of 2.5 mM EDTA and a rapid cooling down to 4ºC.

Preparation of CYP cDNA standards. Standard reference samples of each CYP cDNA were prepared by PCR amplification run to saturation (between 32 and 35 PCR cycles), using the appropriate primers. The resulting cDNAs were purified by column chromatography (High pure PCR product purification kit), and eluted with TE buffer. The samples showed a unique band in agarose electrophoresis. The DNA content was determined by picogreen fluorescence as described above.

Measurement of human CYPs by RT-PCR.. The reverse transcriptase (RT) reaction mixture consisted of 20 µl of 1x reverse transcriptase buffer, 10 mM DTT, 500 µM dNTPs, 3 µM oligo d(T)14 primer, 60 U Rnase OUT and 250 U RTase H. To this mixture, 1 µg of RNA total and 0.01ng of luciferase mRNA was added. The reaction was allowed to proceed for 60 min at 42ºC, followed by a 5 min heating at 95ºC and a rapid cooling on ice. The cDNA was stored at-20ºC until use.

The PCR reaction was conducted in small vessels each one containing 4 µl of appropriately diluted cDNA (problems or standards) and 36 µl of reaction mixture (20 mM Tris-HCl buffer, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 50 µM of each deoxynucleotide triphospate, 1U Taq DNA polymerase and 0.2 µM of each specific primer, final concentration). After 4 min at 94ºC, amplification was performed by 28-30 PCR cycles of 40 sec at 94ºC, 45 sec at 60ºC, 50 sec at 72ºC and a final extension of 4 min at 72ºC.

As schematized in Figure 1 three different amounts of each of the CYP standards and

aproppiate dilutions of the sample to be tested were used in each PCR assay. If two dilutions of the sample, three standards, and a negative control are used for each CYP determination in a 96 well termocycler up to 10 CYPs can be simultaneously determined in 2 samples per run (each CYP: 2 samples x 2 dil + 3 standard + 1 negative control= 8 PCR tubes).

Appropriate cDNA dilutions were empirically determined to ensure that the PCR amplification of each CYP did not reach saturation. Under these conditions, yield was proportional to the input cDNA (13). To quantify the amplified cDNA, 15 µl of the PCR reaction were diluted with TE buffer and stained with picogreen at a 1:400 final concentration. Fluorescence was measured in a micro-plate spectrofluorimeter at 485 nm Excitation 538 nm Emission (14-16). A calibration curve was constructed with known amounts of λ-DNA. Fluorescence was linear in the range 0-90 ng/well.

The concentration of specific cDNAs in samples was estimated from the amount of amplified product measured and the amplification ratio (cDNA content of standards before and after PCR amplification).

RESULTS

Design of specific primers and PCR amplification of human CYPs. Primers were designed using an specific primer analysis software (Oligo, 4.0) and the sequences analyzed by FASTA in the EMBL database. The primers were selected in order to meet three requirements: a) to have similar melting temperatures, b) to match specific, non-conserved regions of each CYP, c) to render amplified cDNA fragments of similar sizes, and d) to flank intronic sequences (except for CYP 1A1 and β-Actin) to exclude that genomic DNA is not contaminating cDNA samples. Oligonucleotide PCR primers were chosen for all major human CYPs (1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5), β-Actin, and luciferase cDNAs. The sequence of both forward and reverse primers, the expected sizes of

the PCR-amplified DNA, and the annealing temperatures are listed in Table I.

To check the suitability of the primers and the uniqueness of the PCR amplified product, a sample of cDNA from a human liver and a small amount of luciferase were amplified simultaneously using the various primers as described in Methods. The PCR-amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. As shown in Figure 2, all amplified CYPs yielded single bands of the expected size (Table I). The amplification of β-Actin, and luciferase, gave also a band of the predicted size.

Under the assay conditions selected, the PCR amplification was exponential, as shown in Figure 3. Indeed, after 30 cycles, there was a linear relationship between the amount of standard CYP added and the resulting amplified product.

Determination of the efficiency of the reverse-transcription step. A possible source of variability in RT-PCR is the efficiency of the reverse-transcriptase step. In order to estimate the inter-assay variability, a known amount of luciferase mRNA was added as internal standard and subsequently reverse transcribed. The resulting luciferase cDNA was amplified by PCR, and the final concentration was determined fluorimetrically by picogreen staining. The initial concentration of luciferase cDNA was determined by interpolating in a curve as described in Methods. Based on the data for luciferase mRNA, the efficiency of the reverse transcription was estimated.

Another key point in reverse transcription is to determine whether low and high amounts of mRNA are transcribed with the same efficiency. To address this point, we carried out assays in samples containing variable amounts of luciferase mRNA (0.001 to 1 ng of per assay) in the presence of an excess of non human liver RNA (1 µg). As shown in Figure 4, low or high amounts of luciferase mRNA were reverse transcribed with similar efficiencies despite the great excess of external RNA.

The RT-step shows little intra-assay variability but significant inter-assay variability (Fig. 5) which is likely due to the sample itself. Average RT efficiency was 13.0±5.9% (n=14). The use of the internal mRNA standard (luciferase) allows the normalization of the RT efficiency values among experiments and, hence, the correct estimation of an specific mRNA content (Table II).

Quantitation of CYP mRNA in liver and cultured human hepatocytes. To show the suitability of this procedure to quantify human CYP in liver tissue and cells, RNA was isolated either from human liver biopsies or cultured human hepatocytes, reverse-transcribed and amplified as described above to quantify the ten most relevant human CYPs. Using the internal standard for the RT step and appropriate CYP cDNA standards, the results can be expressed as number of mRNA molecules per µg of total RNA. CYP3A4 and 2E1 (Table III) were the most abundant CYP messagers in liver cells (average 2.5 and 1.7 x 10^8 molecules mRNA/ µg total RNA respectively). CYPs 1A1, 1A2, 2A6, 2B6, 2D6, 3A5 were approximately tenfold less abundant, while 2D6 showed the lowest number of copies (1.2 x 10^6 molecules mRNA/µg total RNA).

Using the same experimental approach, the induction of CYP was investigated in human hepatocytes incubated 48 h with 3-methylcholanthrene, phenobarbital or rifampin (Fig. 5). Consistent with previous reports, we found that exposure of human cultured hepatocytes to 3-methylcholanthrene resulted in a significant increase in both 1A1 and 1A2 mRNAs. Final content of 1A2 and 1A1mRNAs were 530.7 x 10^5 and 337.5 x 10^5 molecules per µg RNA, respectively. Phenobarbital treatment of hepatocytes resulted in a 4.1 fold increase of the 2B6 mRNA content (from 1.8 to 7.4 x 10^5 molecules per µg RNA) and also significant induction of CYPs 3A4, 3A5 and 2A6. Rifampin treatment resulted in a 10.4 fold increase of 3A4 mRNA (from 2.3 to 23.9 x 10^5 molecules per µg RNA), 5.8 fold of 3A5 mRNA, 3.6 fold of 2B6 and 1.9 fold increase of 2A6.

DISCUSSION

RT-PCR techniques are rapid, specific, and sensitive allowing the detection of very low number of mRNA copies that are not detectable by other analytical approaches such as northern blotting. These features had made RT-PCR an ideal technique for studies where the amount of tissue and mRNA is limited. As little as 1µg of total RNA permits an efficient qualitative analysis of the expression of many genes. However, quantitative measurements by PCR techniques are not straightforward, due to the fact that minor variations in reaction conditions (i.e. extraction, reverse transcription) are greatly magnified during the amplification step. The simplest way to minimize such variations is to express the data as a ratio between the specifically amplified cDNA and another gene showing no changes in expression, which is then taken as internal reference, i.e. β-Actin (17). Experimental conditions must guarantee that the assays have not surpassed the exponential phase of amplification. In this way it is possible to provide information on the relative, but not absolute, RNA content.

The competitive RT-PCR (11) is based on the co-amplification of the specific target sequence, together with known amounts of a competing internal standard in the same reaction tube. Both specific template and internal standard are selected to share primer recognition sites but can be distinguished by size or restriction enzyme analysis. This procedure that requires multiple assays and hence time and labor work, relies its accuracy on the assumption that the two sequences are co-amplified with the same efficiency.

The aim of this study was to define experimental conditions for a reliable quantitative RT-PCR assay for the ten most relevant human CYP mRNAs in cell and tissue samples, in one single PCR run. To achieve this goal, primers (Table I) and amplification conditions (Fig. 2 and Fig. 3) were selected to assure the simultaneous exponential amplification of the

selected genes and to produce unique amplification products, despite their large degree of homology of some of them. Second, appropriate external controls were introduced to minimize differences in the RT-stage, to estimate the RT efficiency and allow absolute quantitation. Under the conditions used, low and high abundant mRNAs were reverse transcribed with the same efficiency. Third, a simple procedure was set up allowing the fluorimetic quantitation of the amplification product in microplates. Finally, by the use CYP cDNA standards containing known amounts of DNA it is possible to express results in terms of mRNA molecules per analyzed sample.

The RT step represents an important source of variability because of differences in reaction efficiency. This problem was solved by using an internal control consisting in a known amount of luciferase mRNA which was reverse transcribed together with the other mRNAs of the sample. The experiments summarized in Table II revealed that the normalization with the internal RT standard corrected the variability among samples.

To assess the reproducibility of the assay we measured the mRNA content of the same samples in several experiments conducted at intervals of more than two months. Small variations were found (Table III) that did not show any appreciable bias related to the CYP or type of sample.

This assay was used to determine the content of CYP mRNA in human liver (Table III), and the results obtained are in agreement with those reported previously by Andersen et al. (18) who found 3.4±2.4 for 1A2, 1.8±1.9 for 2A6/7, 3.0±4.0 for 2D6, 67.6±44.1 CYP 2E1, and 47.9±66.8x10^6 for 3A4/5 molecules per µg of total RNA. The high variation in mRNA CYP abundancy between different individuals or the different procedences or treatments of the biopsies, which is known to affect mRNA abundancy, might account for the small differences in the results.

The consistency of the results obtained are further supported by comparing their

abundance relative to β-Actin mRNA. Schweifkl et al. (19) found in 17 human samples a CYP1A1/β-Actin ratio ranging from 0.006 to 0.126 and a CYP1A2/β-Actin ratio between 0.052 and 1.267 what implies a CYP1A2/CYP1A1 between 10 to 27. In the three samples analyzed in our work the ratio, this time estimated from absolute mRNA CYP contents, was 9.0, 12.7 and 15.4.

The PCR assay here reported was used to monitor the induction of CYPs by 3-methylcholanthrene, phenobarbital and rifampin, three well known inducers of drug metabolizing enzymes. As shown in Figure 5, incubation of human hepatocytes with 3-methylcholanthrene resulted in CYP 1A1, CYP 1A2 mRNAs increases. Phenobarbital induced 2B6, 3A4, 3A5 and 2A6 4.1, 13.1, 4.4 and 3.7 fold respectively. Rifampin caused CYPs 3A4, 3A5, 2B6 and 2A6 increase (10.4, 5.8, 3.6 and 1.9 fold, respectively).

In summary, the RT-PCR method present in this paper is a highly sensitive and specific procedure for quantitative measurements of human CYPs. It allows the measurement of high and low abundant CYPs and can be used both with cultured cells and small liver samples. The technique is reproducible and gives consistent results with those previously published. The application of this method may result in a drastic reduction of the time needed for the secreening of drugs acting as enzyme inducers in human hepatocytes.

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FIGURE LEGENDS

FIG. 1. Schematic representation of the quantitative RT-PCR assay for human CYP determination. Total RNA is extracted from hepatocytes (liver or cultured) and quantified by 260 absorbance and ribogreen. A known amount of the synthetic RNA is added, before performing RT with 1µg of RNA. The cDNA obtained is diluted as required in each case and PCR amplification is carried out in parallel with different amounts of the reference standards and a negative control. The DNA obtained in each case is quantified by picogreen fluorescence. The PCR reaction conditions were determined in such a way that the PCR was always in exponential phase and a single PCR product was obtained.

FIG. 2. Agarose gel analysis of the PCR amplified products. PCR products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. The primers and reaction conditions selected granted unique and specific amplification products of the expected size.

FIG. 3. Range of exponential-phase amplification of CYPs. Known amounts of CYP standards were PCR amplified for 30 cycles under the conditions described in materials and methods. The concentration of the amplified DNA was determined by fluorescence and plotted versus the initial amount of cDNA.

FIG. 4. Reverse-transcription efficiency of low and high concentrated RNAs. Variable amounts of luciferase RNA (1 to 10^{-3} ng per reaction tube) were reverse transcribed in the absence (open bars) or presence of an excess of 1µg RNA (closed bars). The luciferase

cDNA formed was PCR amplified, and quantified as described in M&M, to determine the reverse-transcribed luciferase mRNA and, hence, the efficiency of the RT step.

FIG 5. Intra and inter-assay RT efficiency. Samples of liver RNA containing 0.01ng of luciferase mRNA were reverse transcribed as described in Material and Methods. The luciferase cDNA formed was PCR amplified and quantified to determine the RT efficiency in three different experiments. The RT reaction efficiencies of 14 independent experiments (open bars) and one sample reverse transcribed in 4 different tubes at the same time are represented (closed bars).

FIG. 6. Measurement of CYP mRNA levels in control and xenobiotic-induced human hepatocytes by RT-PCR. Primary cultured human hepatocytes were incubated with 2μM 3-methylcholanthrene (MC) (Panel A), 1 mM phenobarbital (PB) (Panel B) or 50 μM rifampin (Rif) (Panel C) for 48h. Closed bars corespond to controls and open bars to induced hepatocytes. Total RNA was extracted, reverse-transcribed and amplified by PCR. Based on the amount of the amplified cDNA fragment and its molecular weight, the number of mRNA molecules was calculated. Data shows mean values of three measurements ± standard error of the mean.

TABLE I

Oligonucleotide PCR primers for human P-450 enzymes and β-Actin.

Oligo[a] name	Oligonucleotide sequences 5´to 3´	Fragment size (bp)	Anneling temp. (ºC)
CYP 1A1 FP	TCC AGA GAC AAC AGG TAA AAC A	371	5 5.7
CYP 1A1 RP	AGG AAG GGC AGA GGA ATG TGA T		
CYP 1A2 FP	AAC AAG GGA CAC AAC GCT GAA T	453	58.6
CYP 1A2 RP	GGA AGA GAA ACA AGG GCT GAG T		
CYP 2A6 FP	AGG CTA TGG CGT GGT ATT CA	521	58.3
CYP 2A6 RP	ACT CCG TGT TGG GGT TCT TC		
CYP 2B6 FP	ATG GGG CAC TGA AAA AGA CTG A	283	57.8
CYP 2B6 RP	AGA GGC GGG GAC ACT GAA TGA C		
CYP 2C9 FP	ACA GAT AGT GAA ATT TGG AC	403	60
CYP 2C9 RP	TTG CAC AGT GAA ACA TAG GA		
CYP 2C19 FP	ACA GAT AGT GAA ATT TGG AC	277	60
CYP 2C19 RP	TTC ATG CCT TTC TCA GCA GG		
CYP 2D6 FP	CTA AGG GAA CGA CAC TCA TCA C	289	58.2
CYP 2D6 RP	CTC ACC AGG AAA GCA AAG ACA C		
CYP 2E1 FP	ACA GAG ACC ACC AGC ACA ACT	580	55.0
CYP 2E1 RP	ATG AGC GGG GAA TGA CAC AGA		
CYP 3A4 FP	CCT TAC ACA TAC ACA CCC TTT GGA AGT	382	55.3
CYP 3A4 RP	AGC TCA ATG CAT GTA CAG AAT CCC CGG TTA		
CYP 3A5 FP	CCC AGT TGC TAT TAG ACT TGA	679	52.8
CYP 3A5 RP	GGG GCA CAG CTT TCT TGA AGA CCA		
β-Actin FP	CGT ACC ACT GGC ATC GTG AT	452	58.7
β-Actin RP	GTG TTG GCG TAC AGG TCT TTG		
Luciferase FP	TAC ACC CGA GGG GGA TGA TAA AC	439	56.1
Luciferase FP	CTC TTT TTC CGT CAT CGT CTT TC		

[a] oligo= oligonucleotide, FP= forward primer, RP= reverse primer.

TABLE II

Inter assay variation of β-Actin mRNA content in human liver (molecules mRNA/ µg total RNA)

RT assay	RT efficiency (%)	molec βAct/µg RNA (measured)	molec βAct/µg RNA (corrected)
1	15.2	$2.2x10^6$	$1.5x10^7$
2	18.3	$2.6x10^6$	$1.4x10^7$
3	18.1	$2.3x10^6$	$1.3x10^7$
4	5.0	$0.95x10^6$	$1.9x10^7$
Mean	14.15	$2.0\pm0.7x10^6$	$1.5\pm0.3x10^7$

TABLE III

Hepatic P-450s mRNA profiles in liver and in 4h cultured human hepatocytes. (molecules mRNA/ µg total RNA).

CYP	1A1	1A2	2A6	2B6	2C9	2C19	2D6	2E1	3A4	3A5
Liver A	$(21\pm3)x10^5$	$(170\pm10)x10^5$	$(16\pm2)x10^5$	$(170\pm10)x10^5$	$(1100\pm100)x10^5$	$(33\pm2)x10^5$	$(55\pm5)x10^5$	$(2200\pm10)x10^5$	$(1800\pm100)x10^5$	$(31\pm1)x10^5$
Liver B	$(9.4\pm0.1)x10^5$	$(120\pm10)x10^5$	$(3.8\pm0.3)x10^5$	$(11\pm1)x10^5$	$(100\pm20)x10^5$	$(29\pm2)x10^5$	$(30\pm1)x10^5$	$(600\pm40)x10^5$	$(3700\pm600)x10^5$	$(32\pm3)x10^5$
Liver C	$(20\pm5)x10^5$	$(430\pm30)x10^5$	$(98\pm4)x10^5$	$(230\pm40)x10^5$	$(1000\pm100)x10^5$	$(81\pm3)x10^5$	$(22\pm2)x10^5$	$(3200\pm700)x10^5$	$(6700\pm110)x10^5$	$(25\pm6)x10^5$
hep 4h	$(7.6\pm1.1)x10^5$	$(360\pm30)x10^5$	$(54\pm5)x10^5$	$(96\pm14)x10^5$	$(310\pm40)x10^5$	$(45\pm3)x10^5$	$(21\pm3)x10^5$	$(2300\pm500)x10^5$	$(2000\pm100)x10^5$	$(17\pm1)x10^5$

REFERENCES

1. Murray, G.I. and D.M. Burke. (1995) *Biochemical Pharmacology,* **50**, 895-903.

2. Spatzenegger, M. and W. Jaeger. (1995) *Drug Metabolism Reviews,* **27**, 397-417.

3. Meyer, U.A. (1996) *J Pharmacokinet Biopharm,* **24,** 449-59.

4. Wrighton, S.A., M. VandenBranden, and B.J. Ring. (1996) *Journal of Pharmacokinetics and Biopharmaceutics,* **24,** 461-473.

5. Gonzalez, F.J. and H.V. Gelboin. (1994) *Drug Metabolism Reviews,* **26,** 165-183

6. Kerremans, A.L.M. (1996) *Netherlands Journal of Medicine,* **48,** 237-243.

7. Pirmohamed, M., N.R. Kitteringtam, and B.K. Park (1994). *Drug Safty,* **11,** 114-144.

8. Wrighton, S.A., VanderBraden, J.C.Stevens, L.Shipley, L.A., Ring, B.J., Rettie, A.E., Cashman, J.R. (1993) *Drug. Metab. Rev.*, **25**, 453-484.

9. Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., Mayer, R.T. (1985) *Biochem Pharmacol,* **34,** 3337-3345.

10. Parkinson, A. and B. Gemzik. (1991) *Methods in Enzymology*, **206,** 233-245.

11. Zimmerman, K. and J.W. Mannhalter. (1996) *Biotechniques*, **21,** 268-279.

12. Gómez-Lechón, M.J., López, P., Donato, T, Montoya, A., Larrauri, A., Giménez, P., Trullenque, R., Fabra, R., Castell, J.V. (1990) *In Vitro Cell. Dev. Biol.*, **26**, 67-74.

13. Murphy, L.D., Herzog, C.E., Rudick, J.B., Fojo, A.T., Bates, S.E. (1990) *Biochemistry,* **29,** 10351-10356.

14. Singer, V.L., Jones, L.J., Yue, S.T., Haugland, R.P. (1997) *Analytical Biochemistry,* **249,** 228-238.

15. Ahn, S.J., Costa, J., Emanuel, J.R. (1996) *Nucleic Acid Research*, **24,** 13, 2623-25.

16. Enger, O., (1996) *Biotechniques*, **21,** 3, 372-4.

17. Chung, I., and Bresnick, E. (1994) *Archives of Biochemistry and Biophysics,* **314,** 75-81.

18. Andersen, M.R., Farin, F.M., and Omiecinski, C.J. (1998) *DNA and Cell Biology*, **17,** 231-238.

19. Schweikl, H., Taylor, J.A., Kitareewan, S., Linko, P,. Nagorney, D., and Goldstein, J.A. (1993) *Pharmacogenetics*, **3**, 5, 239-49.

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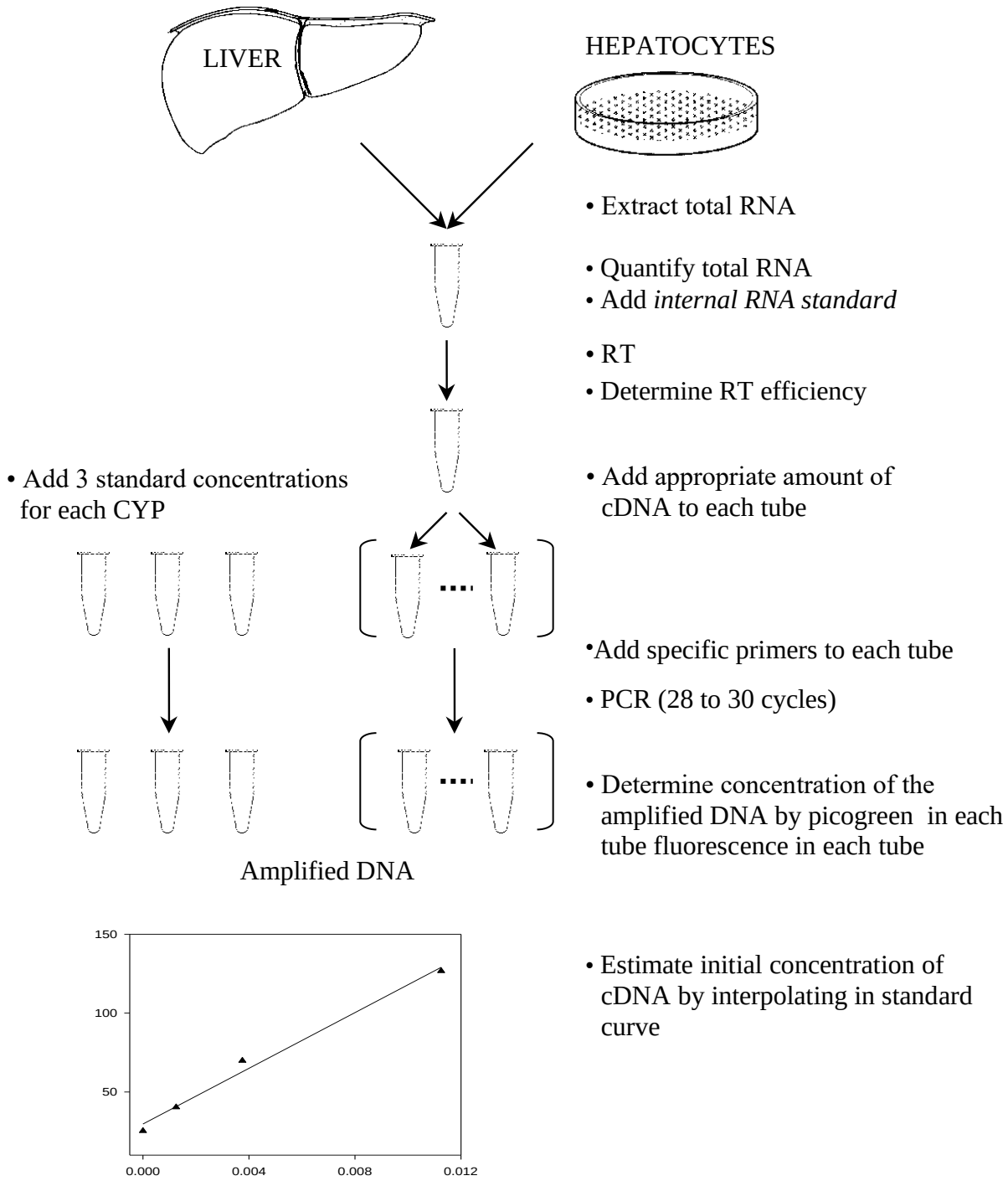

Figure 1
Rodriguez-Antona et al, *Quantitative RT-PCR measurement of human CYP 450 mRNA.*

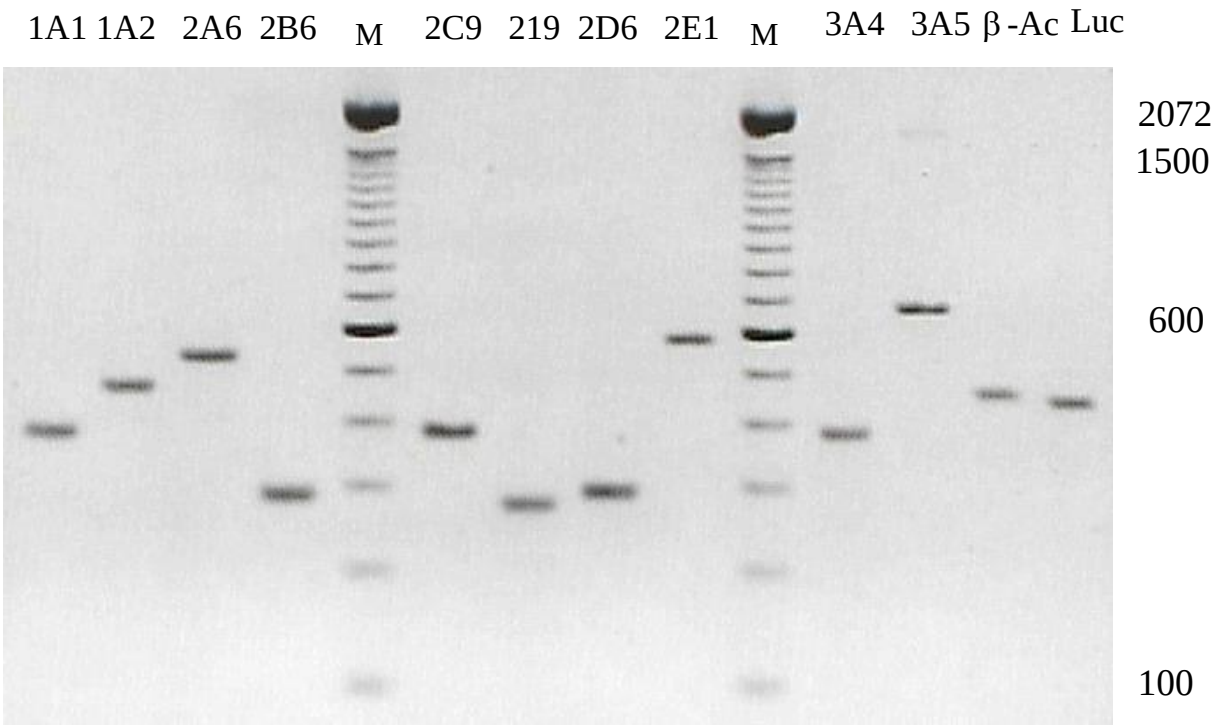

Figure 2
Rodriguez-Antona etal, *Quantitative RT-PCR measurement of human CYP 450 mRNA.*

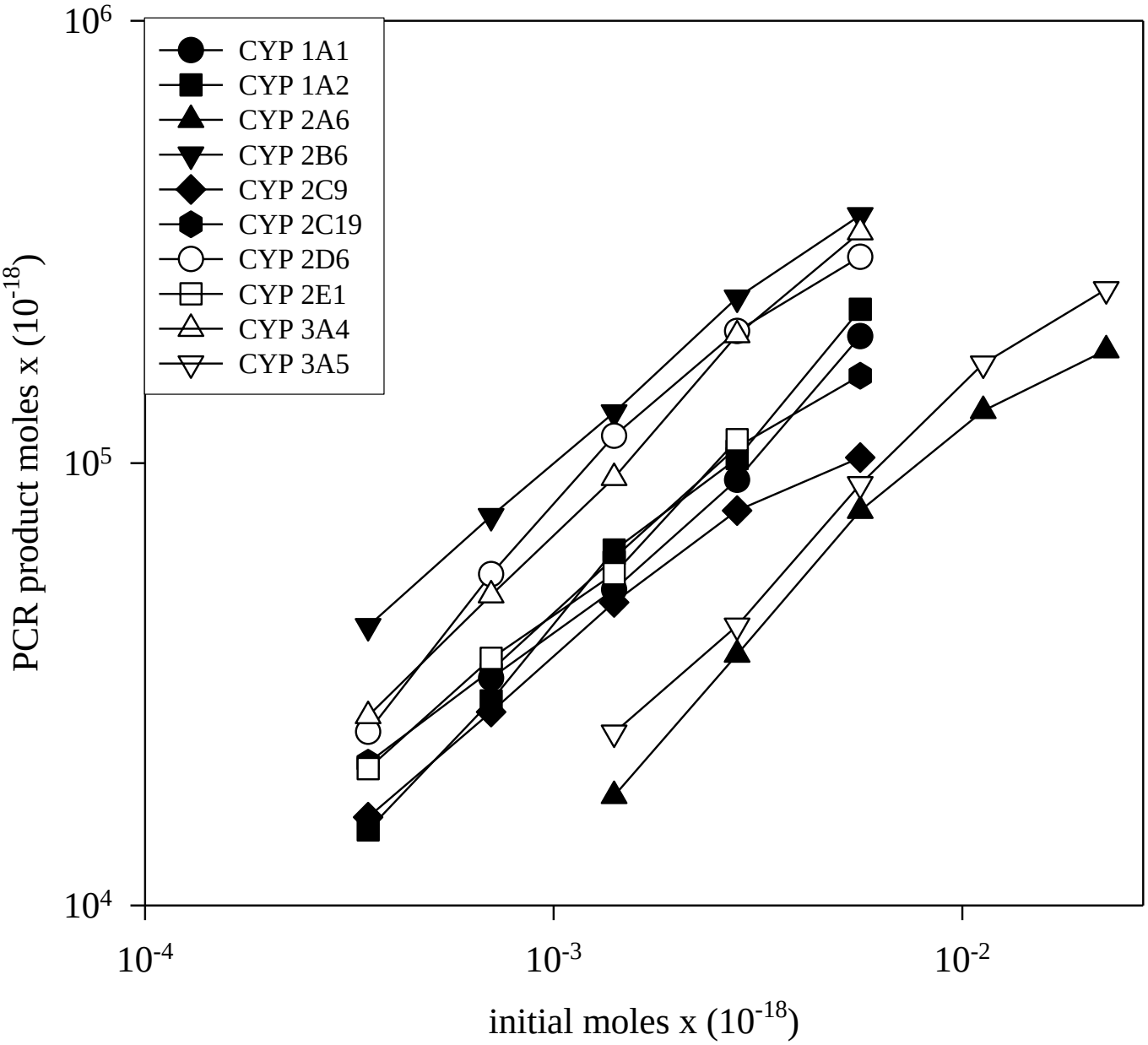

Figure 3
Rodriguez-Antona et al, *Quantitative RT-PCR measurement of human CYP 450 mRNA.*

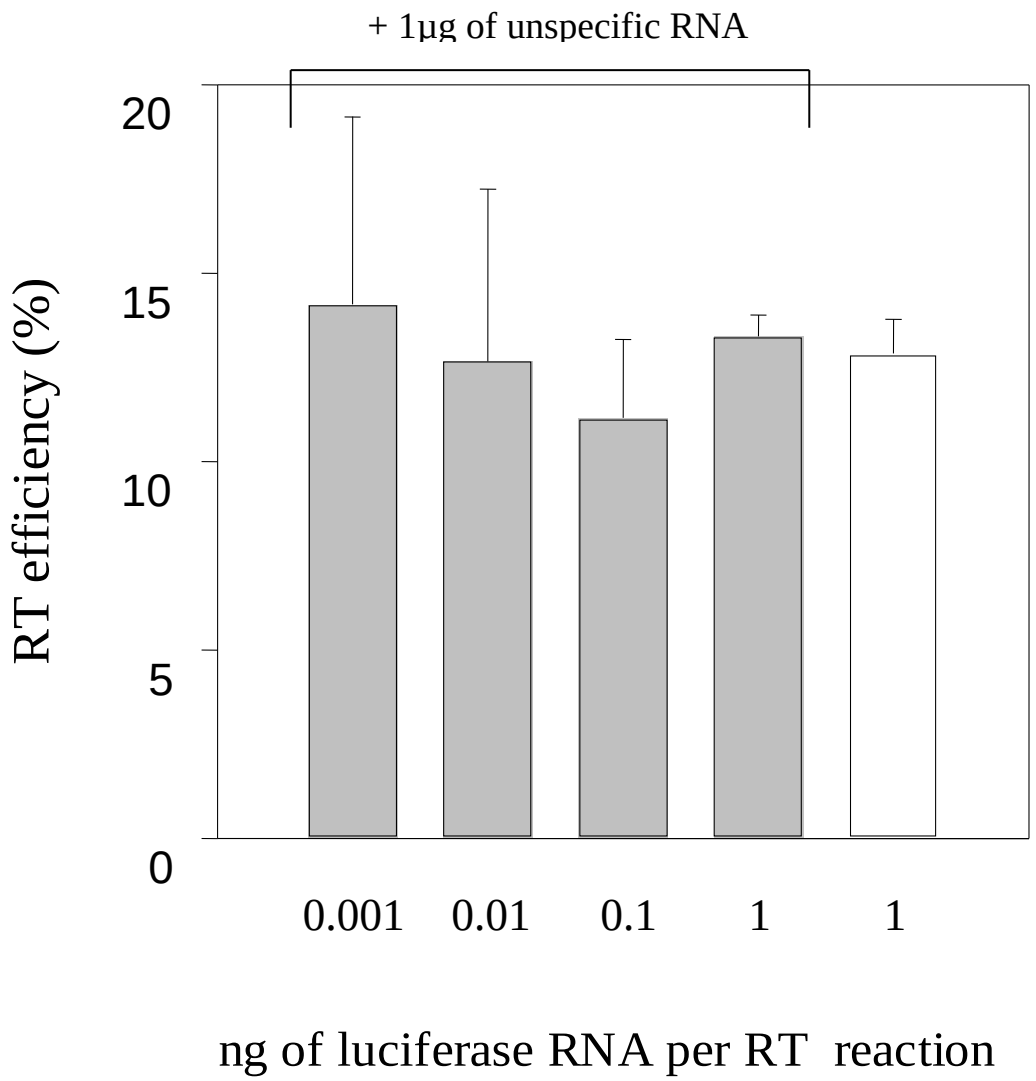

Figure 4
Rodriguez-Antona et al, *Quantitative RT-PCR measurement of human CYP 450 mRNA.*

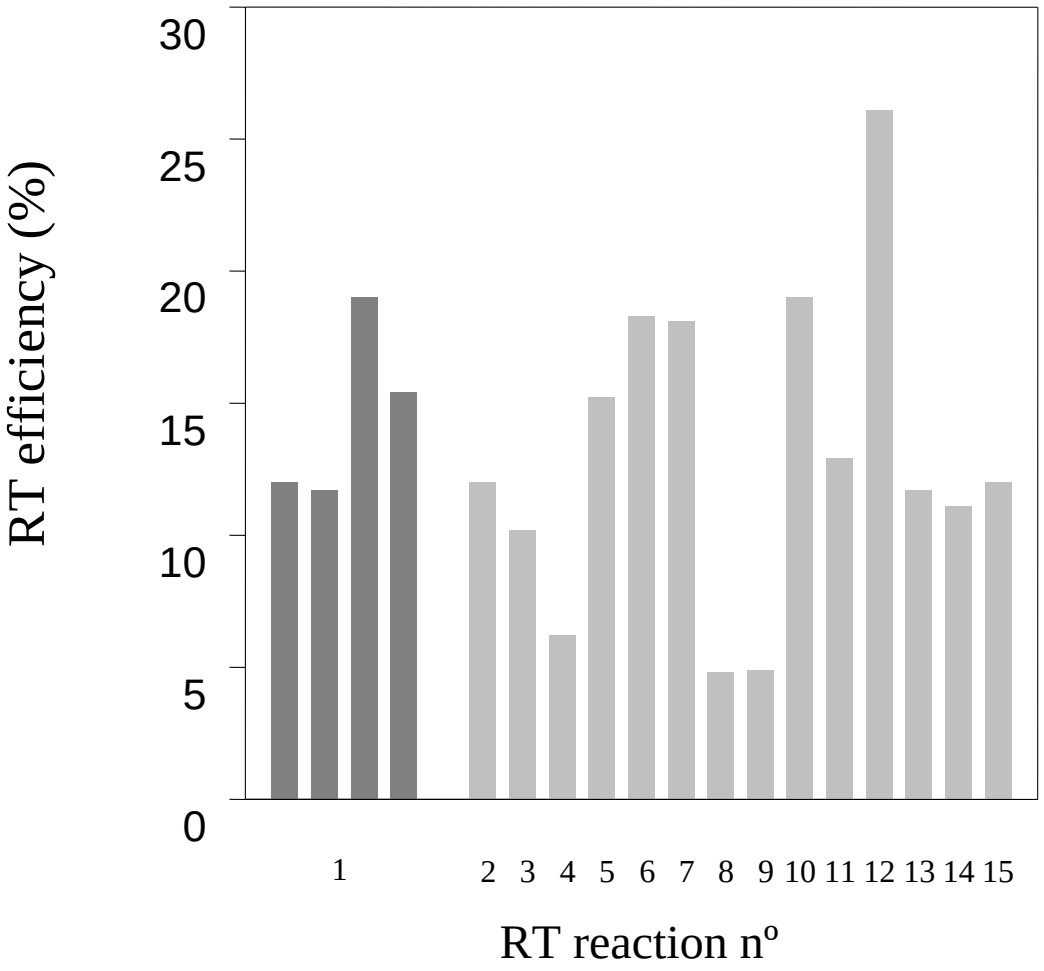

Figure 5
Rodriguez-Antona et al, *Quantitative RT-PCR measurament of human CYP 450 mRNA.*

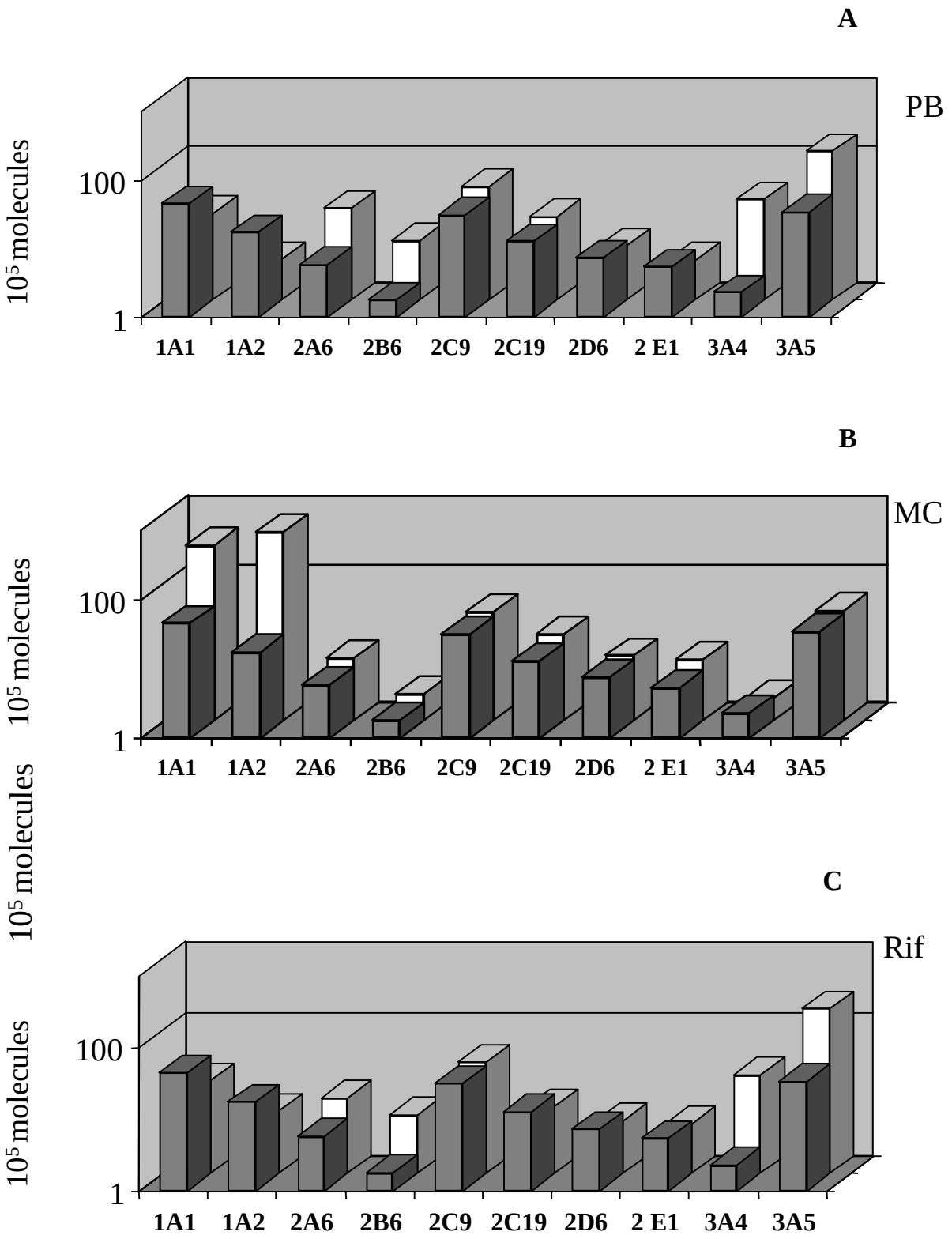

Figure 6
Rodriguez-Antona et al, *Quantitative RT-PCR measurement of human CYP 450 mRNA.*