



# *High-content Gene Expression Analysis with TRAC*

## Introduction

Years of intensive global gene expression studies have provided an abundance of genome-wide expression data, enabling identification of gene expression signatures for diverse biological states such as disease states, patient responses or toxicological responses. The subsequent need is to analyze focused gene sets from large sample amounts cost and time efficiently for research, drug screening and diagnostic purposes.

TRAC (Transcript analysis with the aid of affinity capture) is a novel hybridization and bead based assay enabling multiplex mRNA target detection simultaneously from large sample numbers. The functionality of TRAC has been shown in a number of applications including molecular toxicology, gene expression based monitoring of biotechnical processes, cell-based cancer marker gene screening, siRNA research and pathway studies.

# TRAC technology

The TRAC method enables rapid quantification of focused gene sets from a large number of samples. In TRAC analysis transcript levels are measured directly from lysed cells, without need for RNA purification or cDNA conversion. Each chosen gene of interest is recognized by a specific complementary fluorophore-labeled probe. A pool of probes with different lengths or type of label is added to each sample with a hybridization buffer.

Biotin-oligo-dT is used to capture targets from their polyA tails. Hybridization of probes and transcripts takes place in solution and the purification of the hybrids is performed in 96-well microtiter plate format with an automated sample processor apparatus.

The probe-transcript complexes are captured by streptavidin coated magnetic beads. Unbound material is washed off and probes are eluted off from the beads.

The hybridization, capture, washing and elution are completed in 2-3 h with little hands-on time. The probe pools in each sample are separated by capillary electrophoresis, which resolves the probes according to size and label. Separated probes are quantified by their fluorescence signal. The maximum capacity of one TRAC assay with 96 samples and 30 chosen targets yields 2880 transcript levels. [1,2] (Figure 1.)

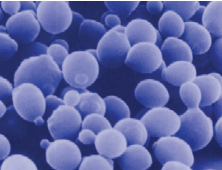
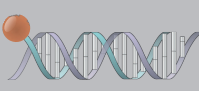
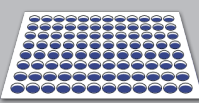
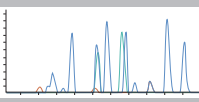
1	Sampling & Cell Lysis		<b>Objective:</b> Expose RNA to allow testing w/o extraction or amplification
2	Hybridization		<b>Objective:</b> Capture RNA, in solution
3	Automated Sample Treatment		<b>Objective:</b> Isolate target RNA probes for measurement
4	Separation/ Detection		<b>Objective:</b> Measure expression levels for each target RNA of interest

Figure1. Assay procedure

# User benefits

TRAC assay enables high-content gene expression analysis with the following user benefits.

**Simplicity:**

- Direct use of cell lysates - No RNA extraction
- No cDNA conversion required
- Simple assembly of new custom made gene sets
- Easy implementation
- Simple experiment set-up

**Accuracy:**

- Degradation of RNA avoided during the process
- Control genes part of multiplex mixture – normalization of results

**Robustness:**

- High-throughput sample processing in 96-well plate format using automated sample processing
- Multiplex detection of target genes (5-30-plex)
- Rapid assay protocol with short hands-on time

**Reproducibility:**

- Intra and inter assay CVs ca.10 %

# TRAC application example

Human cytochrome P450 expression screening in primary hepatocytes

## Objective

The cytochrome P450 superfamily (CYP) is a large and diverse group of enzymes that function in oxidation of organic substances. CYPs are the major enzymes involved in drug metabolism, accounting for ~75% of the total metabolism of currently used drugs. The activity of CYPs is affected by numerous environmental and genetic factors, making drug metabolism exceedingly variable and individualistic. Better understanding of CYP activity helps to predict the toxicity and metabolism of drugs in the early stages of drug development.

Analyzing the CYP activity in cultured primary hepatocytes is a commonly used method in preclinical pharmacological studies. The enzyme activity is traditionally evaluated by protein based activity assays, which are time-consuming and require large amount of sample material. TRAC offers an efficient high-throughput method to measure the mRNA levels of multiple CYP genes simultaneously.

In this study, TRAC was used to analyze the expression of 10 CYP genes from primary hepatocytes. The induction of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP2D6, CYP2C8 and CYP2E1 mRNAs by 9 different chemicals, was studied from three donors. RT-qPCR was performed from some of the targets to validate the assay.

## Materials and methods

The cells were cultured in 48-well plates with  $1.7 \times 10^5$  cells per well. The cells were exposed to test chemicals or vehicle control (DMSO) in triplicate wells. After a 24h incubation, 100  $\mu$ l of PlexPress lysis buffer was added to the wells to prepare the samples for TRAC. Parallel cultures were processed for cDNA synthesis and RT-qPCR analysis.

For TRAC analysis 15  $\mu$ l of the lysates (with approx. 25 000 cells) was used for each hybridization with PlexPress HybeMix containing a pool of the 10 CYP450 genes and the reference genes GAPDH, B2M and SDHA. After a 2h hybridization the samples were processed with an automated magnetic bead processor (KingFisher, ThermoFisher Scientific) and the samples were analyzed with an ABI3730 DNA Analyzer. All steps were performed in 96-well plates.

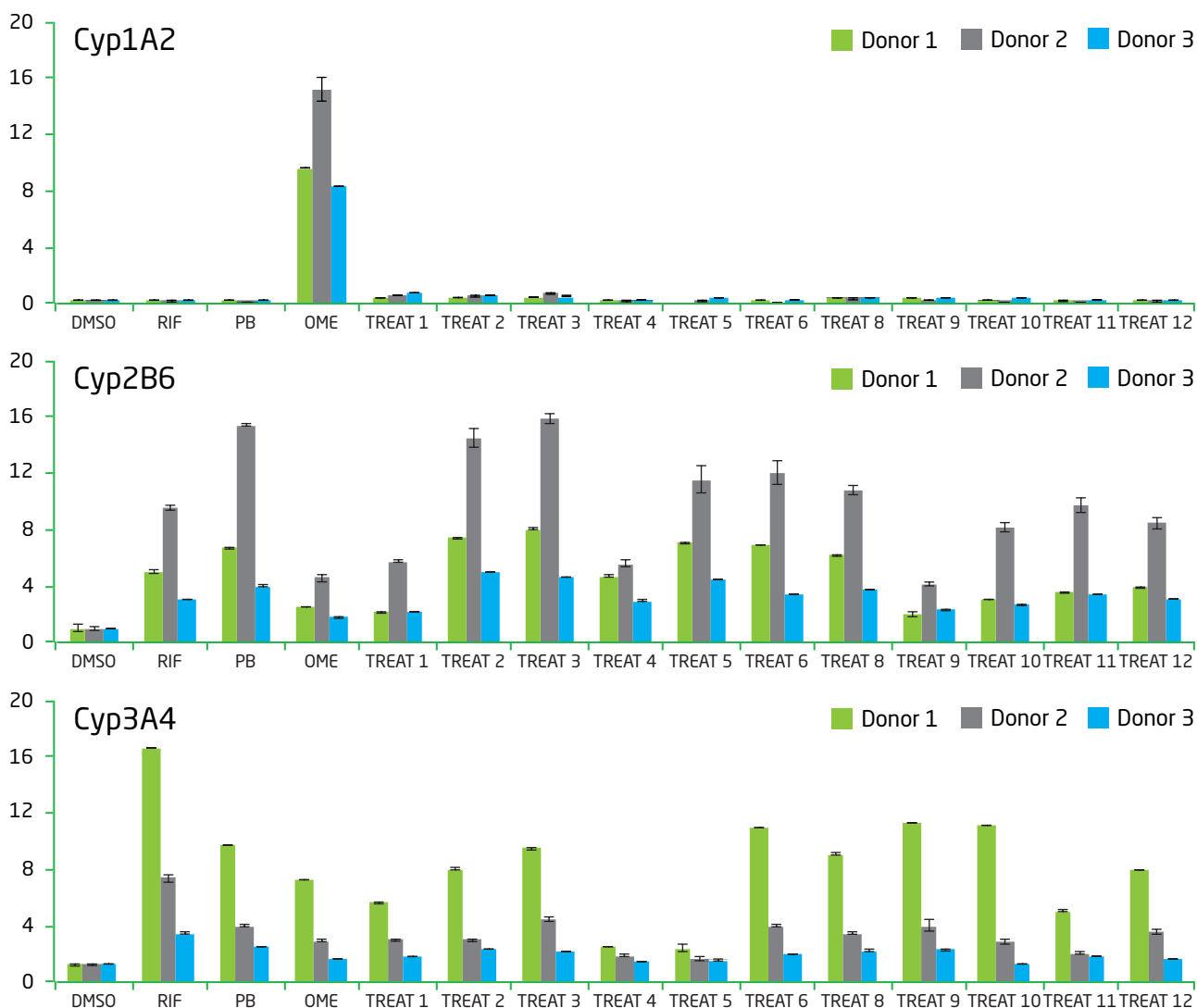
Samples for quantitative RT-PCR were prepared using Cells-to-Ct kit (Ambion). The cells were lysed, treated with DNase and cDNA was synthesized. TaqMan assays from Applied Biosystems were used for CYP1A2, CYP2A6, CYP2B6, CYP3A4, CYP2C9 and CYP2C19. In addition, GAPDH and  $\beta$ -actin were analyzed for normalization of the data.

## Results

### CYP expression screening

CYP3A4, CYP1A2, CYP1A1, CYP2A6, CYP2B6 were the most responsive to used treatments. These targets responded to the known CYP450 inducers rifampicin, omeprazole and phenobarbital as

expected. The induction levels varied between the donors (Fig 2). For other CYP450 target mRNAs the inductions were in general less than two-fold for the used chemical treatments.



**Fig 2. Expression profiles of CYP3A4, CYP1A2 and CYP2B6 analyzed by TRAC in differently treated primary hepatocytes from 3 donors. Error bars represent the standard error of the mean (SEM). Expression levels are presented as fold change to vehicle treatment (DMSO).**

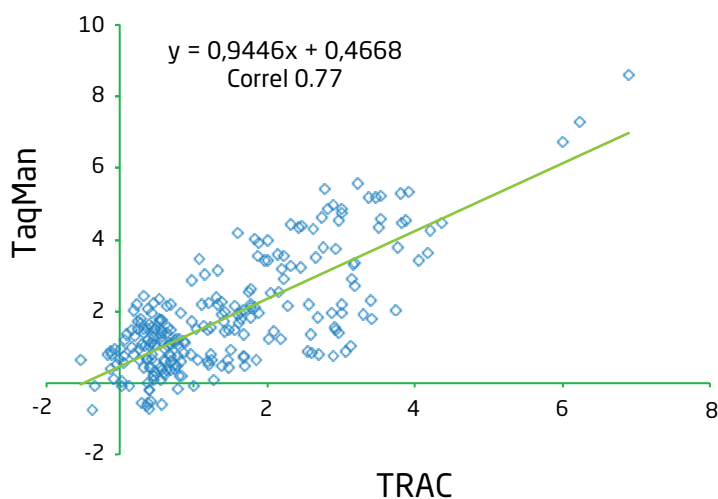
## Comparison between TRAC and TaqMan

CYP450 is a group of genes with highly similar mRNA sequences, posing challenges in terms of the specificity of the used expression method. RT-qPCR is the most widely used gene expression analysis method and is typically used in routine CYP expression analysis. To compare the performance and reproducibility of TRAC and RT-qPCR for CYP450 expression, TaqMan data was generated from hepatocyte samples.

By analyzing 3 donors, 14 different treatments and 6 CYP genes from each sample, altogether 252 data

points were generated with both methods for the comparison. The coefficient of correlation was 0.77 for the TRAC and TaqMan comparison and the slope was 0.94 (Fig 3).

Each data point was an average of three parallel wells that were analyzed with three technical replicates. The coefficient of variation was calculated for the parallel measurements. The table below summarizes the reproducibility comparison between TRAC and TaqMan.

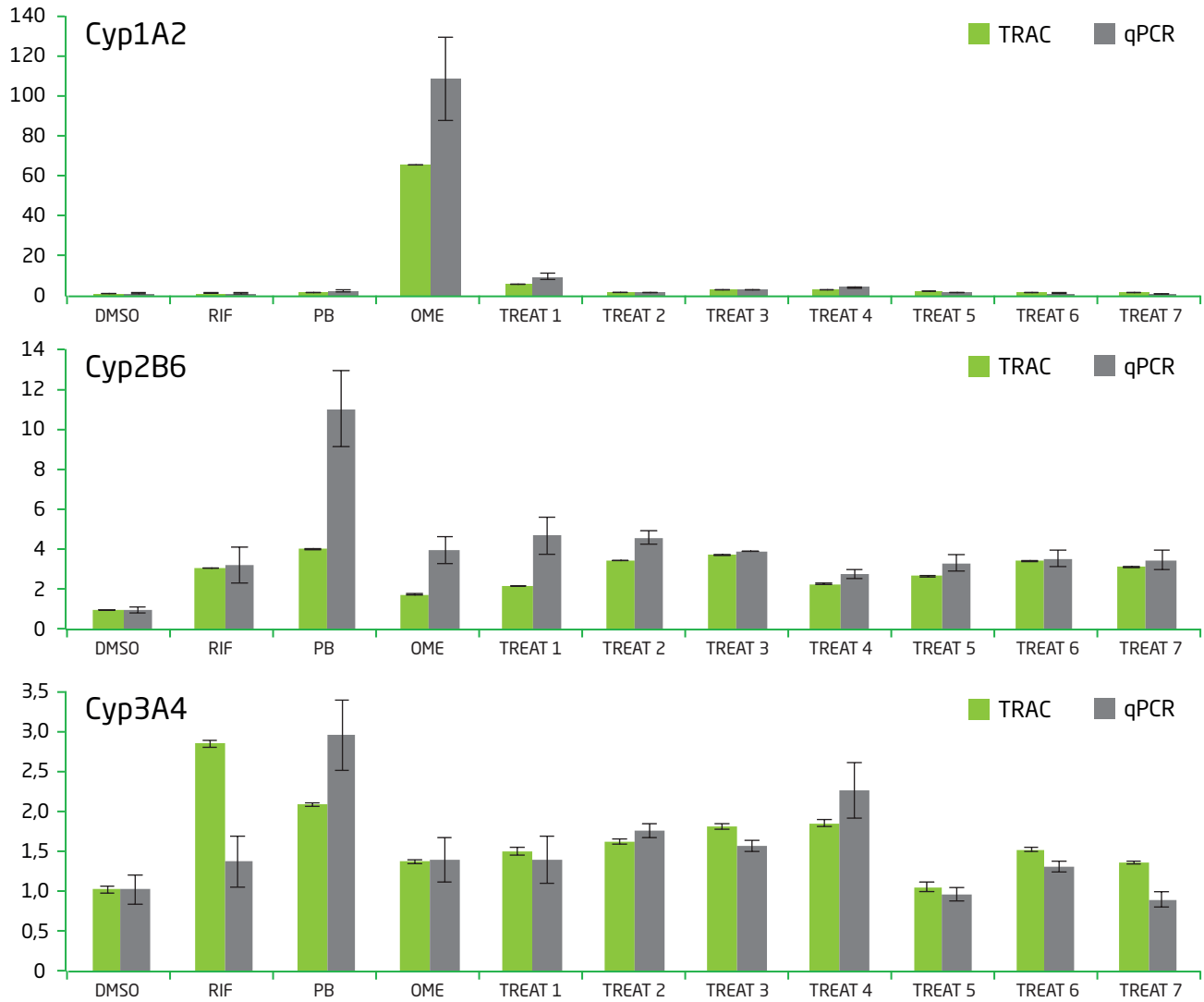


**Fig 3. Fold-change correlation between TRAC and TaqMan assay for 252 data points. Expression profiles of CYP3A4, CYP1A2 and CYP2B6 were analyzed with TRAC and TaqMan in differently treated primary hepatocytes from 3 donors.**

		Donor 1	Donor 2	Donor 3
TRAC	CV% <sup>1</sup>	9,3	9,8	9,8
	SEM% <sup>2</sup>	3,2	4,3	2,3
TaqMan	CV% <sup>1</sup>	43,1	41,5	48,3
	SEM% <sup>2</sup>	12,9	13,6	15,1

1 CV% of data normalized with housekeeping genes

2 SEM/fold-change to control



**Fig 4. The expression of Cyp1A2, Cyp2B6 and Cyp3A4 analyzed by TRAC and RT-qPCR in differently treated primary hepatocytes. Error bars represent the standard error of the mean (SEM). Expression levels are presented as fold change to vehicle treatment (DMSO).**

## Conclusions

TRAC offers a simple and flexible method to measure the expression levels of key CYP genes associated with drug metabolism together with selected housekeeping genes. The performed experiment showed that TRAC can be used directly for crude lysates of primary hepatocytes and the cells cultured in 96-well format are directly applicable as samples for TRAC. The results showed good correlation with quantitative RT-PCR. The results presented here were obtained by performing 432 TRAC reactions (4.5 x TRAC assays). If the same was performed with singleplex qPCR, it would have required 4320 PCR reactions (excluding housekeeper reactions and negative controls).

### The benefits of TRAC over PCR based methods are:

- Direct use of cell lysates without RNA extraction or cDNA conversion
- A set of up to 30 genes can be measured in a single well
- Possibility of including housekeeping genes in the same well with target genes improves the normalization accuracy
- Cost and time advantage resulting from low reagent expenses and short hands on time with few manual steps
- High reproducibility resulting from the avoidance of enzymatic amplification steps and automated sample processing

### Literature

- [1] Rautio JJ et al (2006), Rapid and multiplexed transcript analysis of microbial cultures using capillary electrophoresis-detectable oligonucleotide probe pools. *J Microbiol Methods* 2006, 65:404-416.
- [2] Rautio JJ et al (2008) TRAC in high-content gene expression analysis: applications in microbial population studies, process biotechnology and biomedical research. *Expert Rev. Mol. Diagn.* 8, 379-385.