Detection of Cytochrome P450 Gene Expression in Human Hepatocytes for Pre-clinical and Clinical Trials: Challenges in Validating RT-QPCR Assays in a Regulated Environment

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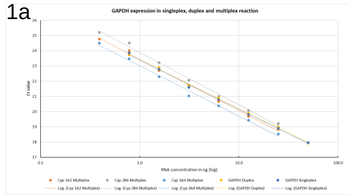
1 INTRODUCTION

It is a regulatory requirement that vectors used in gene therapy or advanced vaccine therapy be tested for integration, biodistribution and persistence prior to any clinical testing.
Established regulations and guidelines are in place for investigating DNA based vectors, whether viral or plasmid and define the parameters for development and validation of assays for the bioanalysis of these therapeutics.
In contrast the specification of assays for the detection of RNA-derived products using relative quantitation is more challenging and scientists have to balance client needs and expectations, regulatory guidelines and their own interests.
We have developed a multiplex RT-qPCR assay for detecting the expression of Cytochrome P450 genes in human hepatocytes and will be moving forward to validate this assay, in compliance with GLP and GCP regulations, to allow its use in Pre-clinical and Clinical Trials.

2 DEVELOPMENT

- Human hepatocytes were stimulated with different concentration of Omeprazole, Phenolbarbitol or Rifapicin to induce CYP expression
- RNA extraction using KingFisher magnetic bead processor
- Gene expression for Beta-2-Macroglobulin (B2M), GAPDH, CYP 1A2, CYP 2B6 and CYP 3A4 were assessed in singlepex, duplex and triplex reaction (Fig. 1 a+b, Fig. 2 a-c)
- CYP 1A2, CYP 2B6 and CYP 3A4 expression in response to the different stimuli are presented in Fig. 3 a-c

1a



1b

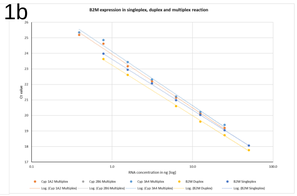


Fig.1: Relative standard curves for (a) B2M and (b) GAPDH run in singleplex, duplex and multiplex reaction .

Fig.2: Relative standard curves for (a) Cyp 1A2, (b) Cyp 2B6 and (c) Cyp 3A4 run in singleplex (blue) and multiplex (orange) reaction.

Fig.3: Expression profile of (a) Cyp 1A2 and (b) Cyp 2B6 and (c) Cyp 3A4 in response to stimulation.

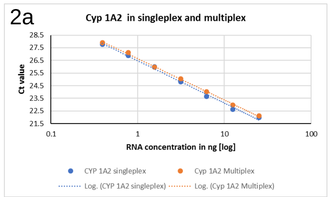
Table 1: Slope and efficiency for Cyp 1A2, Cyp 2B6 and Cyp 3A4

		Singleplex	Multiplex
Cyp 1A2	Slope	-3.351	-3.271
	Efficiency	98.081	102.170
Cyp 2B6	Slope	3.378	-3.371
	Efficiency	97.708	98.008
Cyp 3A4	Slope	-3.489	-3.421
	Efficiency	93.577	96.041

3 RESULTS

- We successfully developed a multiplex assay to investigate CYP expression in the presence of two housekeeping genes (Table 1, Fig. 2 a-c, 3 a-c)
- First assessment show linearity of the assay for each gene over 60-fold dilution series of RNA
- Stimulation of hepatocytes was detectable over a 40-fold dilution series of inducing chemical
- For assay validation linear dynamic range, limits of detection (LOD) and Intra-/ Inter-assay variation need to be assigned (Fig. 4)

2a



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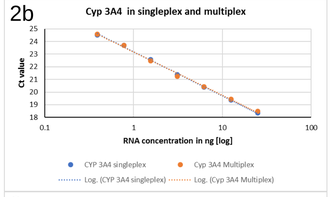
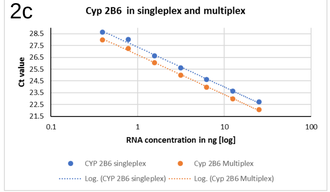
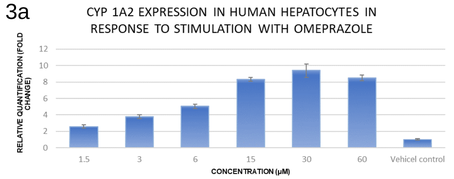


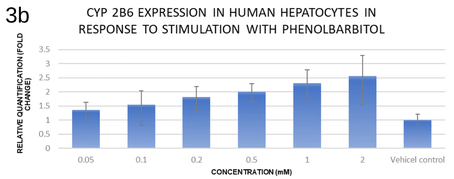
2c



3a



3b



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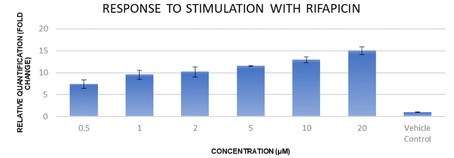


4 VALIDATION

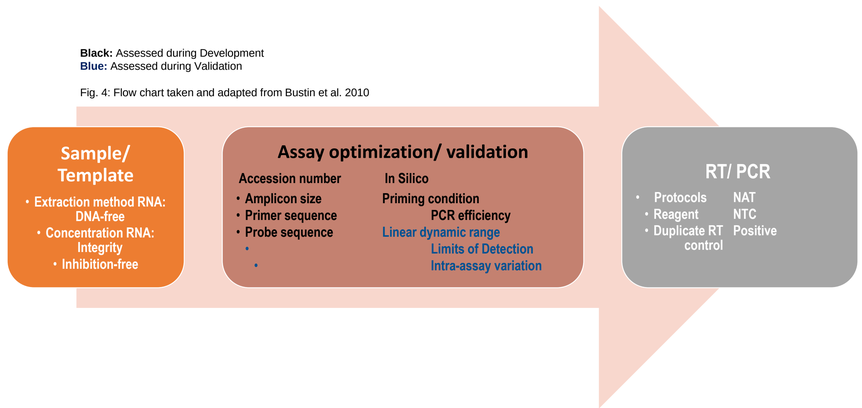
Linear dynamic range:
Dilution series of target-specific RNA, converted to cDNA, across a 2-3 log range will be assessed.
For multiplex assays, the singleplex reaction is run at first and its performance compared to the multiplex reaction.
The Ct values for the individual dilutions points should not vary significantly between singleplex and multiplex reaction.

Inter-/ Intra-assay variation:
Replicates of the same target-specific cDNA in at least two dilutions (1:5 and 1:50, also test for inhibition as difference in Ct should be 3.3-3,4)
Assessment on the same plate for intra-assay assessment and on different plates for inter-assay assessment by at least two analysts.

Limits of Detection:
Linearity based minimum input RNA established to gain qualifiable signal

Black: Assessed during Development
Blue: Assessed during Validation

Fig. 4: Flow chart taken and adapted from Bustin et al. 2010



5 DISCUSSION

Assessment of intra-/ inter-assay variation for repeatability and reproducibility have to be interpreted with care as many factors may influence RNA-based assay, e.g. RT-step in a 1-step RT-PCR.
The assessment of the LOD is not possible as no standard curve and no absolute quantitation is present. Therefore a minimum input of RNA may be assessed where the signal is still detectable.

In general, validating and performing RT-qPCR assays in an industrial environment will not fulfil all demands made by the MIQE guidelines as these would result in higher time and budget expenses which have to be met by either the client or the company. In most cases, the minimum necessary task will be performed to provide the client with the results that will meet regulatory criteria.

6 MATERIALS AND METHODS

- MagMAX Total RNA Extraction kit (KingFisher, Thermo Fisher)
- Quantstudio 7, Applied Biosystems
- TaqPath 1-Step MasterMix with Mustang Purple (ThermoFisher)
- B2M assay, VIC labelled (Thermo Fisher)
- GAPDH assay, JUN labelled (Thermo Fisher)
- Cyp1A2, 2B6 and 3A4 assays, FAM labelled (Thermo Fisher)

References:
Bustin et al. 2010, MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments

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