Normalisation of mRNA Levels Using Expressed *Alu* Repeats (EARs) to Investigate Immunoregulation

Nina Witt[1], Jo Vandesompele[2], Alimuddin Zumla[1], Graham Rook[1] and Jim Huggett[1]*

1: Centre of Infectious Diseases & International Health, Division of Infection & Immunity, UCL, 46 Cleveland Street, London, W1T 4JF, UK
2: Centre for Medical Genetics, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium
*Corresponding author j.huggett@ucl.ac.uk

Aims

- To analyse cytokine mRNA expression changes in dendritic cells treated with the environmental mycobacteria *Mycobacterium vaccae*.
- Investigate the use of expressed *Alu* repeats (EARs) as a normalisation strategy when measuring cytokine mRNA.

Background

Alu repeats are short interspersed nuclear elements (SINEs) that comprise ~10% of the human genome and are frequently expressed in the untranslated regions of mRNAs[1]. Primers designed to amplify the *Alu* consensus sequence of cDNA allow the expression of many different transcripts to be measured at the same time. As there are so many EARs, they could provide a good measure of cDNA and be used to normalise expression data. This can allow similar accuracy to using multiple reference genes with a much simpler strategy[1].

Material & Methods

Primers were designed to amplify the *Alu* consensus sequence: Alu-J-1 (F) 5'-CAACATAGTGAAACCCCGTCT (300 nM) and Alu-J-1 (R) 5'-GCCTCAGCCTCCCGAGTAG (300 nM). qPCR reaction (Fig. 1) was performed using the QuantiTech-SYBR reagents (Qiagen) using the Rotorgene 3000 (15 min 95 °C followed by 45 cycles of 95 °C for 10 secs, 58 °C for 20 secs, 72 °C 30 secs) . Alu-J 1 results where compared with the human acidic ribosomal protein P0 (RPLP0) (Fig. 2)[2].

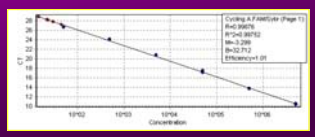

Fig. 1: Dilution series of the Alu-J-1 plasmid. The red points represent the Alu concentration in the NTCs.

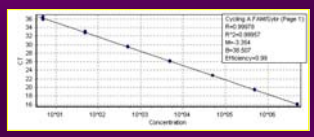

Fig. 2: Dilution series of the RPLP0 plasmid .

PBMCs were isolated from buffy coat using standard procedures and RNA extracted from ~2 × 10^6 PBMCs using RNeasy (Qiagen). RNA was quantified using the Nano drop 1000 and quality assessed using agarose gel electrophoresis. RNA was treated for 30 min with RNase-free DNase (Promega, UK) and reverse transcribed (~25 ng of RNA) using oligo dT primed SuperScript III.

All steps were performed after cleaning the work area and while wearing a mask to avoid contamination (Fig. 4).

Fig. 4: Nina wearing a mask while setting up qPCR reaction to avoid contamination

Results: Problem of contamination from genomic DNA (gDNA)

•cDNA from ~25 ng PBMC RNA contains ~10000 copies of expressed Alu-J-1 (Fig. 5).

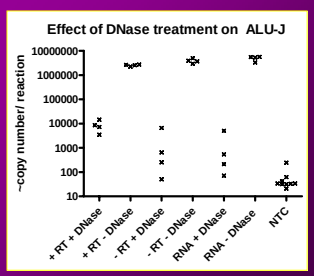

Fig. 5: Effects of DNase treatment on copy number of *Alu* repeats. RNA was reverse transcribed (+RT), the enzyme replaced by water (-RT) or only RNA was added to the reaction (RNA).

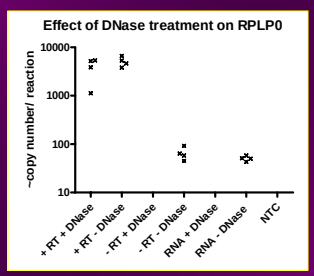

Fig. 6: Effects of DNase treatment on copy number of RPLP0. RNA was reverse transcribed (+RT), the enzyme replaced by water (-RT) or only RNA was added to the reaction (RNA).

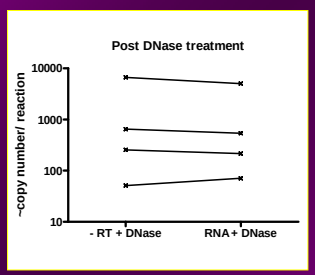

Fig. 7: Copy number of *Alu* repeats after DNase treatment of the extracted RNA. In the reverse transcription reaction the enzyme was replaced by water (-RT) or RNA was added to the reaction without reverse transcription (RNA).

- The RT controls: minus reverse transcriptase (-RT +DNase) or only RNA (RNA + DNase) demonstrate that there is still genomic DNA remaining following DNase treatment.
- DNase is not always working with the same efficiency.
- The variation does not result from the qPCR reaction (Fig. 7).
- The RPLP0 processed pseudogene DNA is completely removed with DNase treatment (Fig. 6).

Results: Problem of contamination from reagents

When performing ALU-J-1 qPCR the negative controls (NTCs) always produce a result at ≤100 copies/reaction (Fig. 1 and 5). We investigated the source of this contamination by exposing 1 ml of water for different durations of time to the air on an open work bench (Fig. 8). We added 5 µl of the water to a 12.5 µl qPCR reaction. This contamination does not appear to be due to air exposure (Fig. 8).

We also investigated if contamination could be removed by treating the mastermix with UV light prior to adding template DNA. Figure 9 illustrates that the measured copy number of Alu-J-1 in the NTCs drops off with longer UV treatment of the mastermix.

But this decrease is partly due to the fact that the UV light reduces the efficiency of the reaction (Fig. 10). UV treatment of 5 min represents a feasible strategy, as the reduction is not detected (Fig. 10) yet contamination is reduced by 10 fold (Fig. 9).

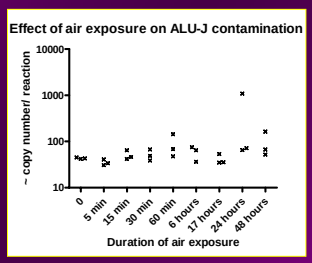

Fig. 8: 1 ml water was exposed for different times to lab air. 5 µl was added to a 12.5 µl qPCR reaction. The copy number of ALU repeats does not increase with the duration of air exposure.

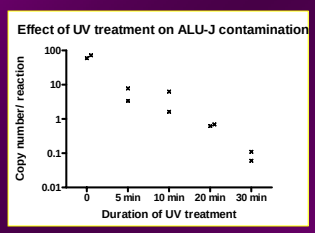

Fig. 9: Following different durations of UV light treatment of the mastermix, primers were added and reaction performed. The measured copy number decreases with longer UV treatment.

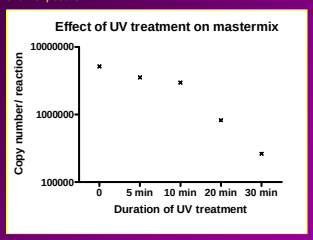

Fig. 10: After UV light treatment of the mastermix, primers and plasmid DNA (5 × 10^6 reaction) was added to the qPCR reaction. The copy numbers measured by qPCR decrease with longer UV treatment of the mastermix, thus the efficiency of the qPCR reaction drops off with longer UV treatment.

Conclusion

- When detecting EARs by qPCR it is essential that DNase treatment is performed to remove the gDNA, however this can lead to variable detection so RT negative controls must always be included.
- EAR detection is also susceptible to contamination that does not appear to be from the laboratory but from a reagent source.
- UV treatment of the mastermix for ~5 minutes can reduce this contamination.

References:
1: Vandesompele et al. Normalisation of gene expression: state of the art and preview on a strategy using expressed Alu repeats, 2nd international qPCR symposium Freising 2005.

2: Dhedah et al. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal Biochem. 2005 Sep 1;344(1):141-3

Acknowledgments:
We gratefully acknowledge the support from the European Commission's Human Resources and Mobility Programme, Marie Curie Research Training Networks.