



# *Fluctuation of commonly used and novel reference genes in time course experiments*

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# Overview

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- **Interested in the administration of hormones**
  - observing the effect on expression of hormone regulated genes over time
  - sampling at several time points over a 24-48 hour period
- **Use prostate and breast cancer cell lines**
  - effects of estradiol (E2) treatment on ZR-75-1 breast cancer cell line; target gene – PR
  - effects of androgen (DHT) treatment on LNCaP prostate cancer cell line; target gene – KLK3 (PSA)
  - Reference genes (RGs) – ALAS1, GAPDH, GUSB, HMBS, HPRT1, MRPL19, RPL32, and RAC1 (novel)



## Protocol

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- **QPCR results can be affected by experimental procedures**
  - Cell plating, harvesting and RNA extraction
- **Plate and treat cells using plugged pipette tips to reduce contamination**
- **RNA extraction: Trizol or Qiagen RNeasy Kit**
  - Visualisation of RNA integrity by agarose gel electrophoresis
- **DNaseI treatment on column**
- **Ensure that RNA has A260/A280 ratio of 2.0**

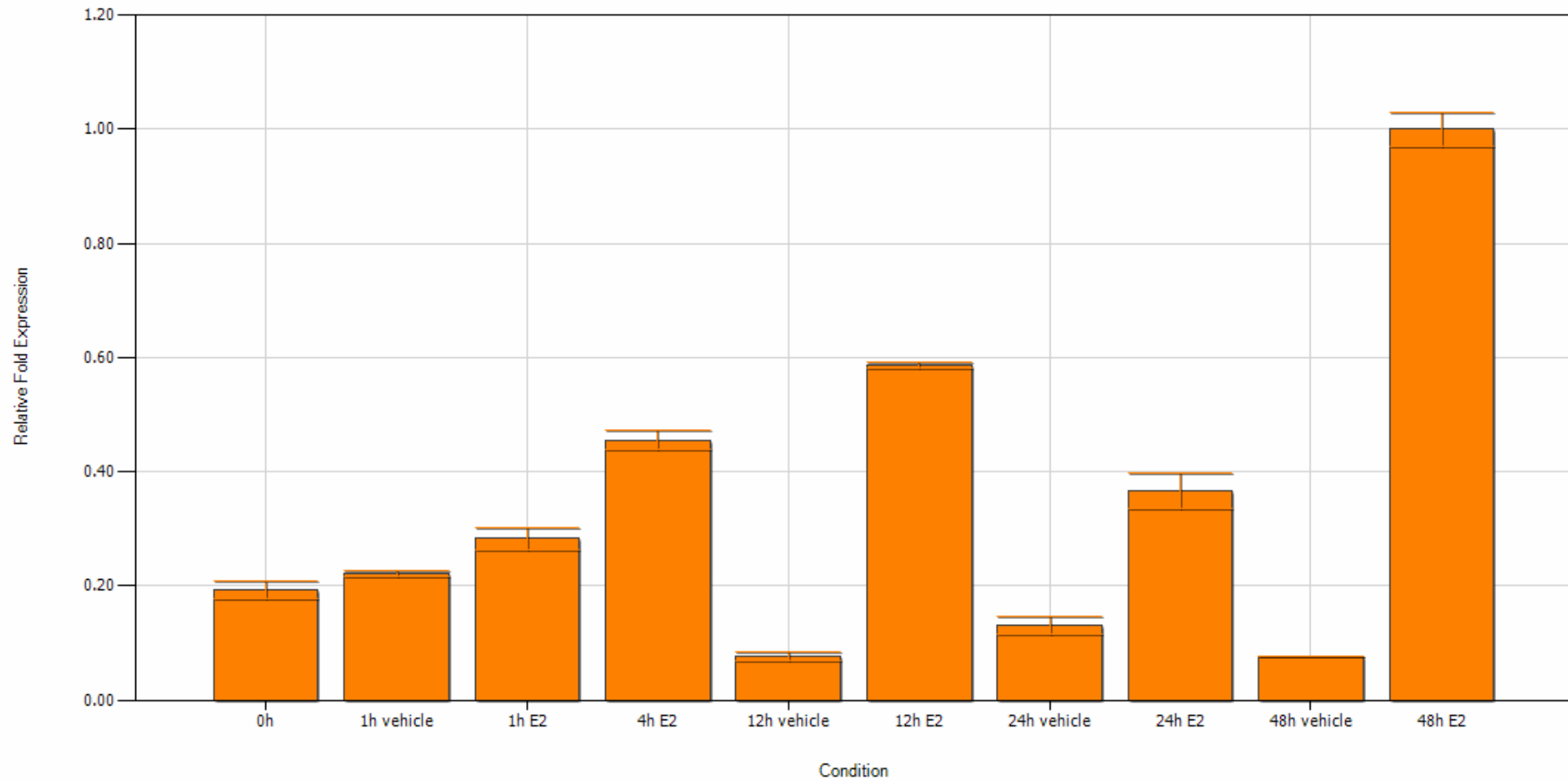


# Protocol

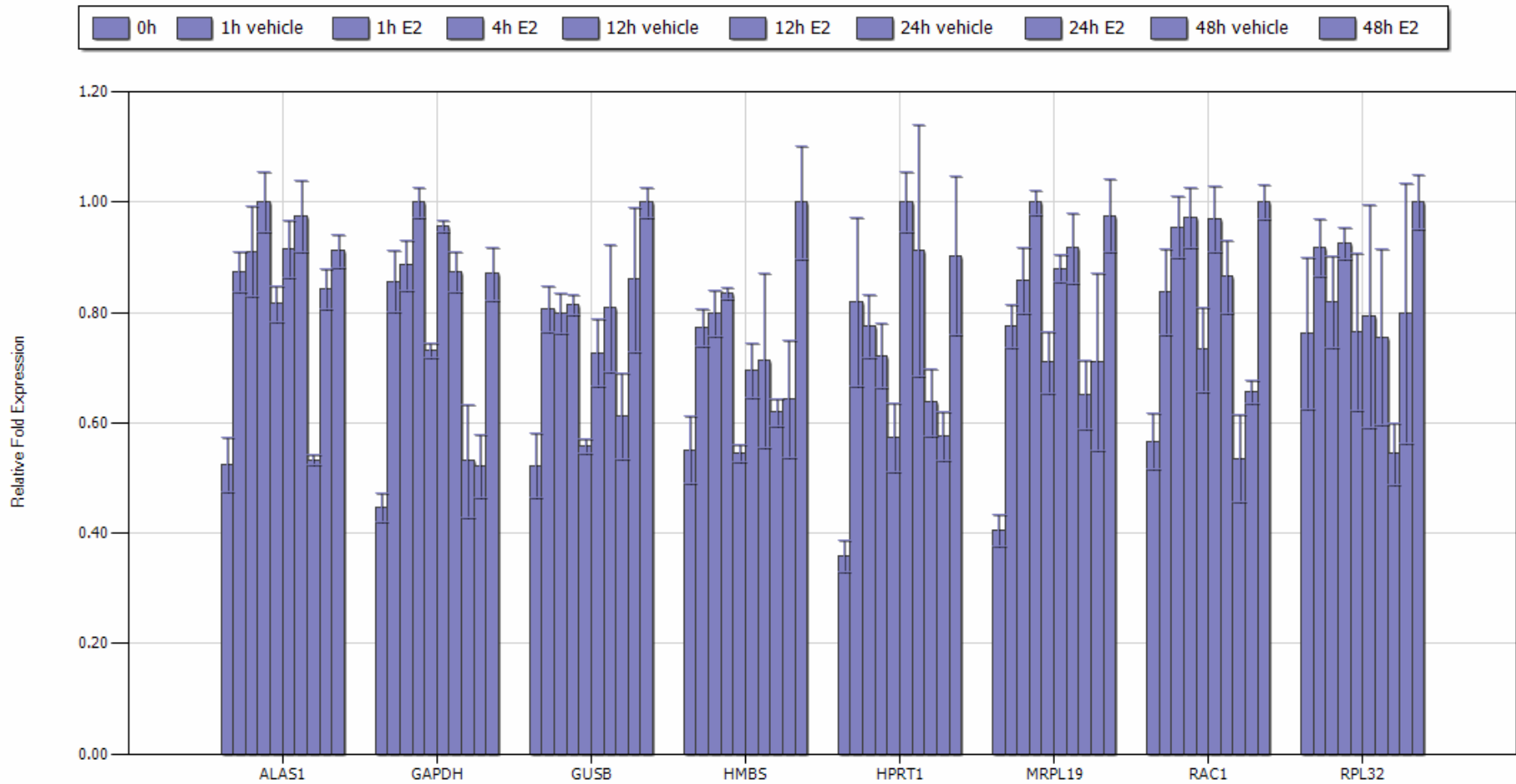
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- **Ensure input RNA concentration in RT reaction is the same**
  - Oligo dT and random hexamers
  - Dilute cDNA after RT reaction – depending on RNA amount in RT (200-400ng)
  - RNA only and RT negative controls
- **QPCR reactions are performed in triplicate**
  - SYBR green
  - Standard curve of Universal Human Breast or Prostate RNA to determine reaction efficiency (1:2, 1:20, 1:200, 1:2000, 1:20,000 standard curve in duplicate)
  - Water controls
- **Analyses**
  - iQ5 – reaction efficiency, gene expression, normalisation of data to number of genes
  - geNorm and NormFinder analysis using Genex

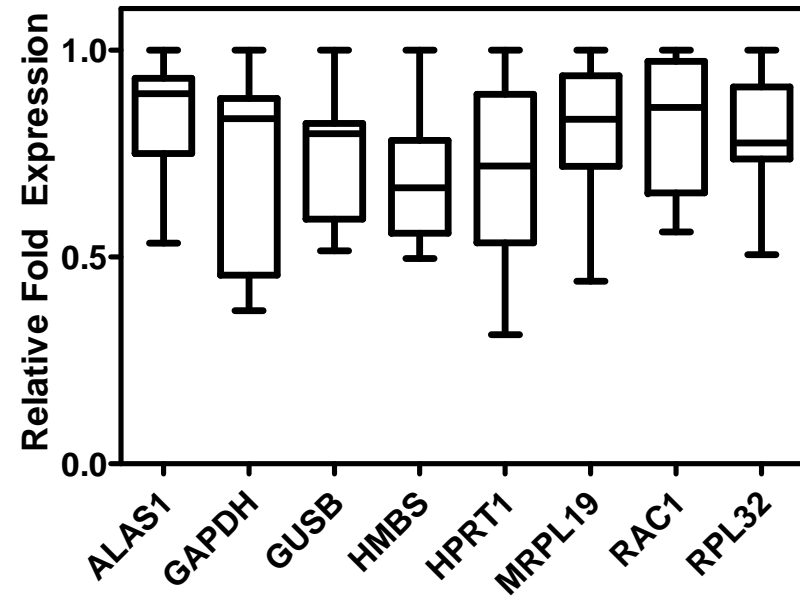
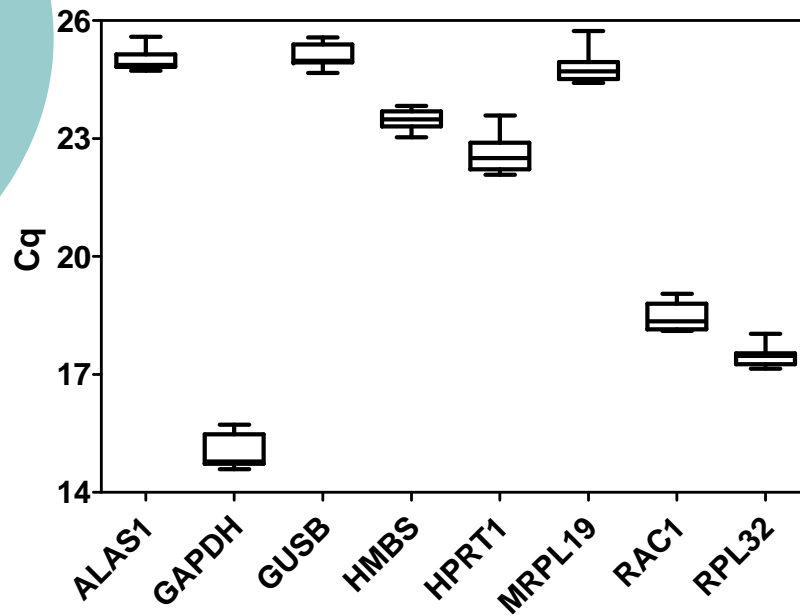
# ZR-75-1 E2 time course - PR



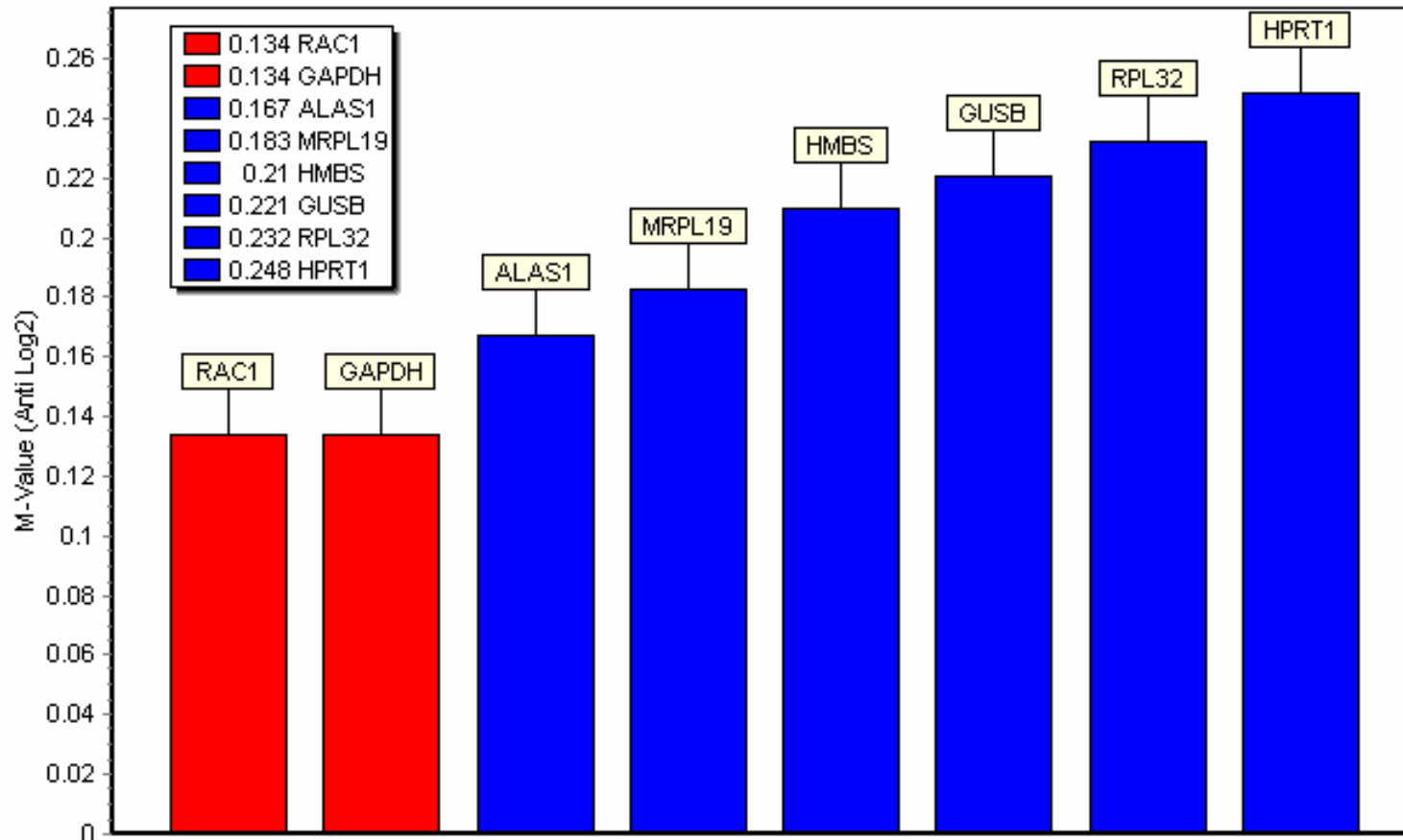
# ZR-75-1 E2 time course - RGs



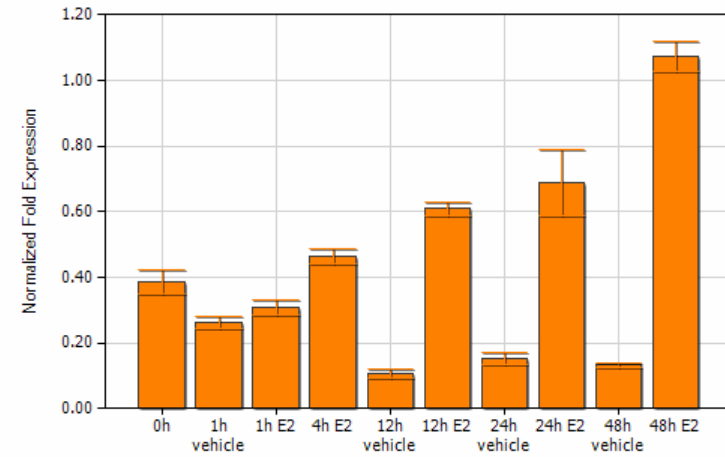
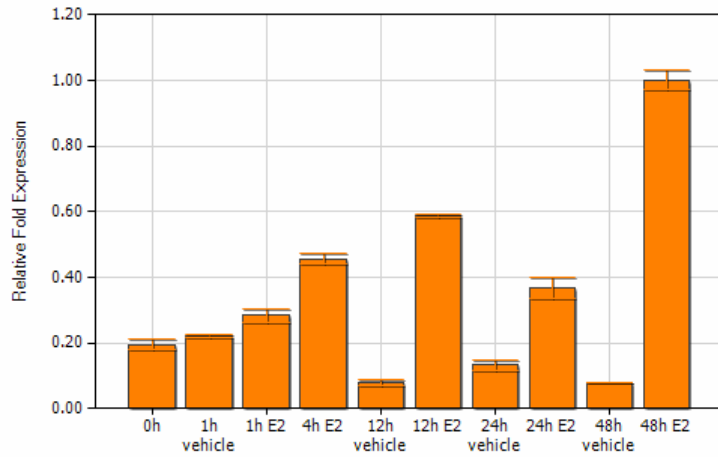
# ZR-75-1 E2 time course - RGs



# ZR-75-1 geNorm analysis

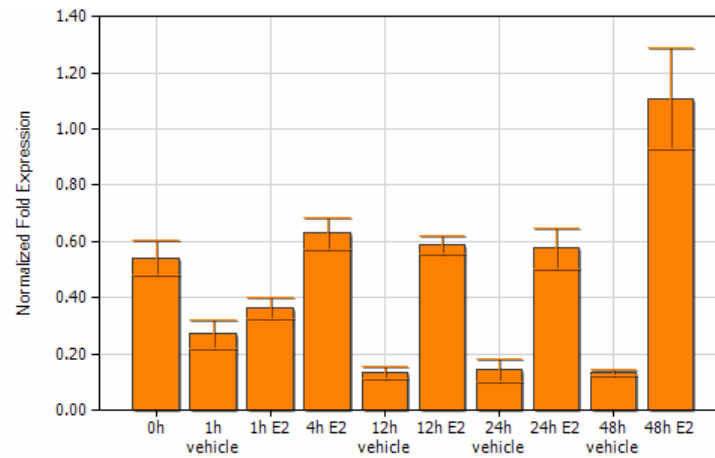


# PR expression



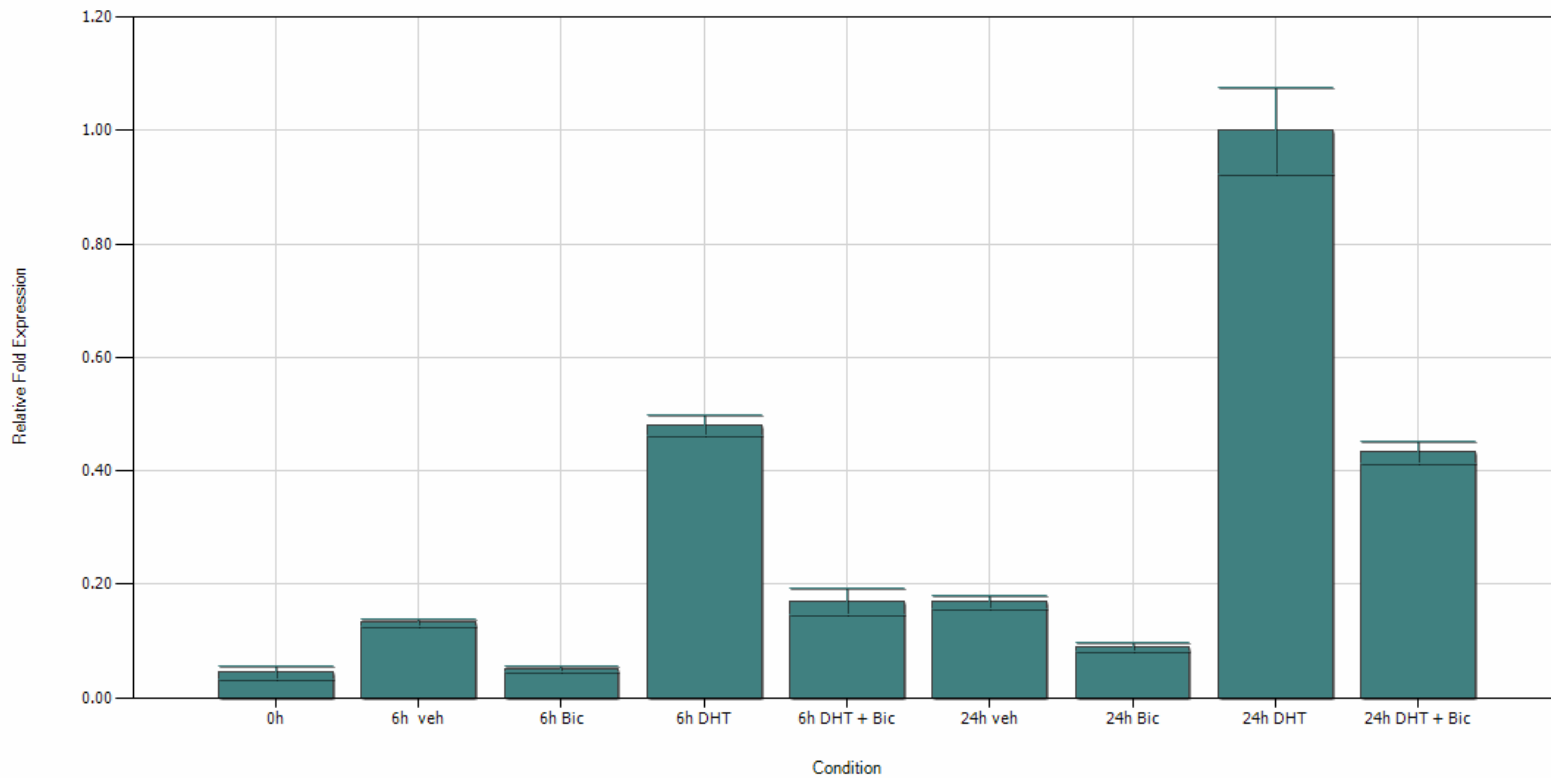
**Non-normalised**

**Normalised to  
RAC1 and GAPDH**

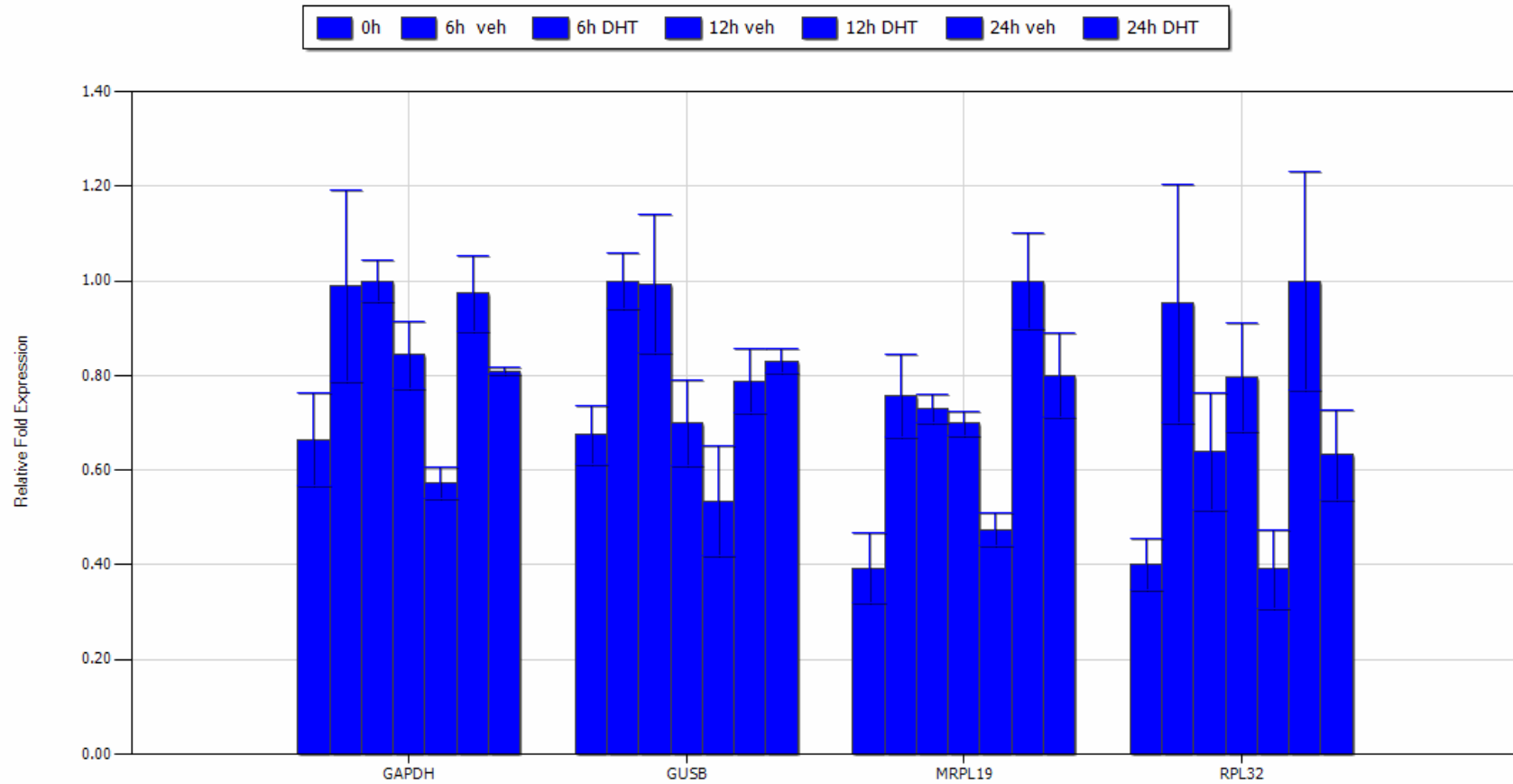


**Normalised to HPRT1**

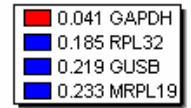
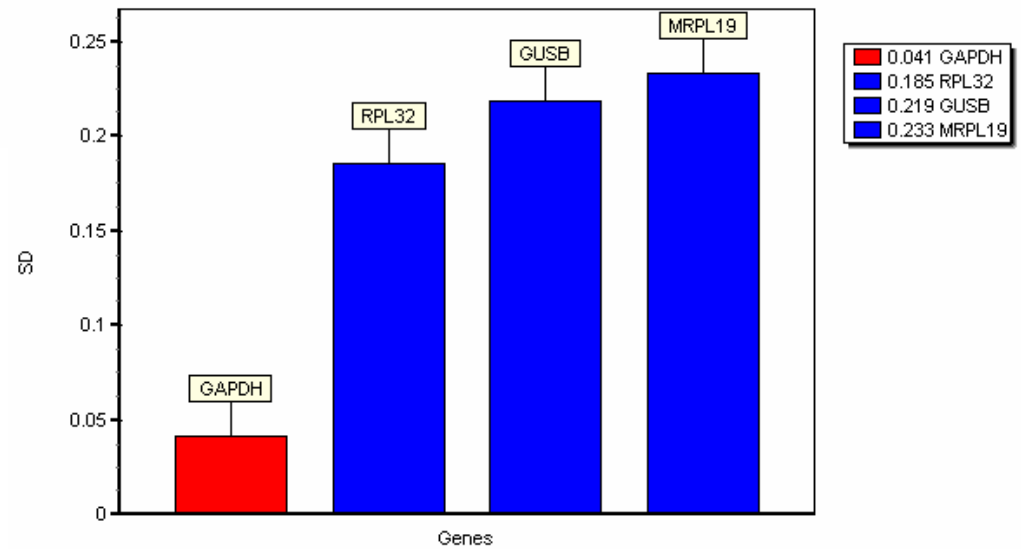
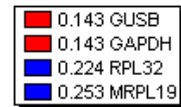
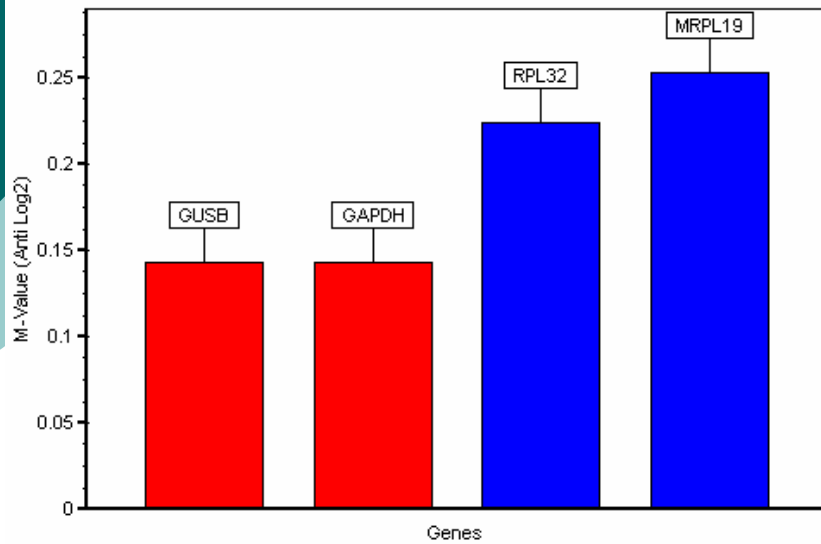
# LNCaP DHT time course – KLK3



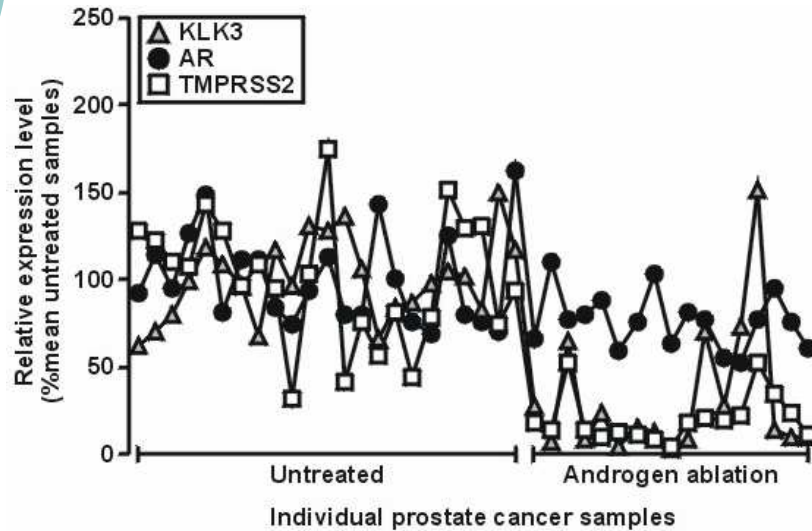
# LNCaP DHT time course - RGs



# geNorm & NormFinder analysis

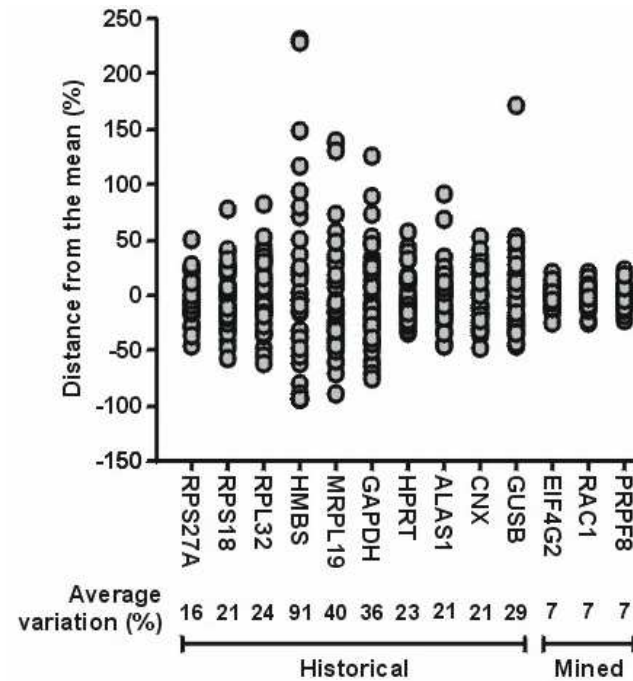


# Data mining to find potential RGs



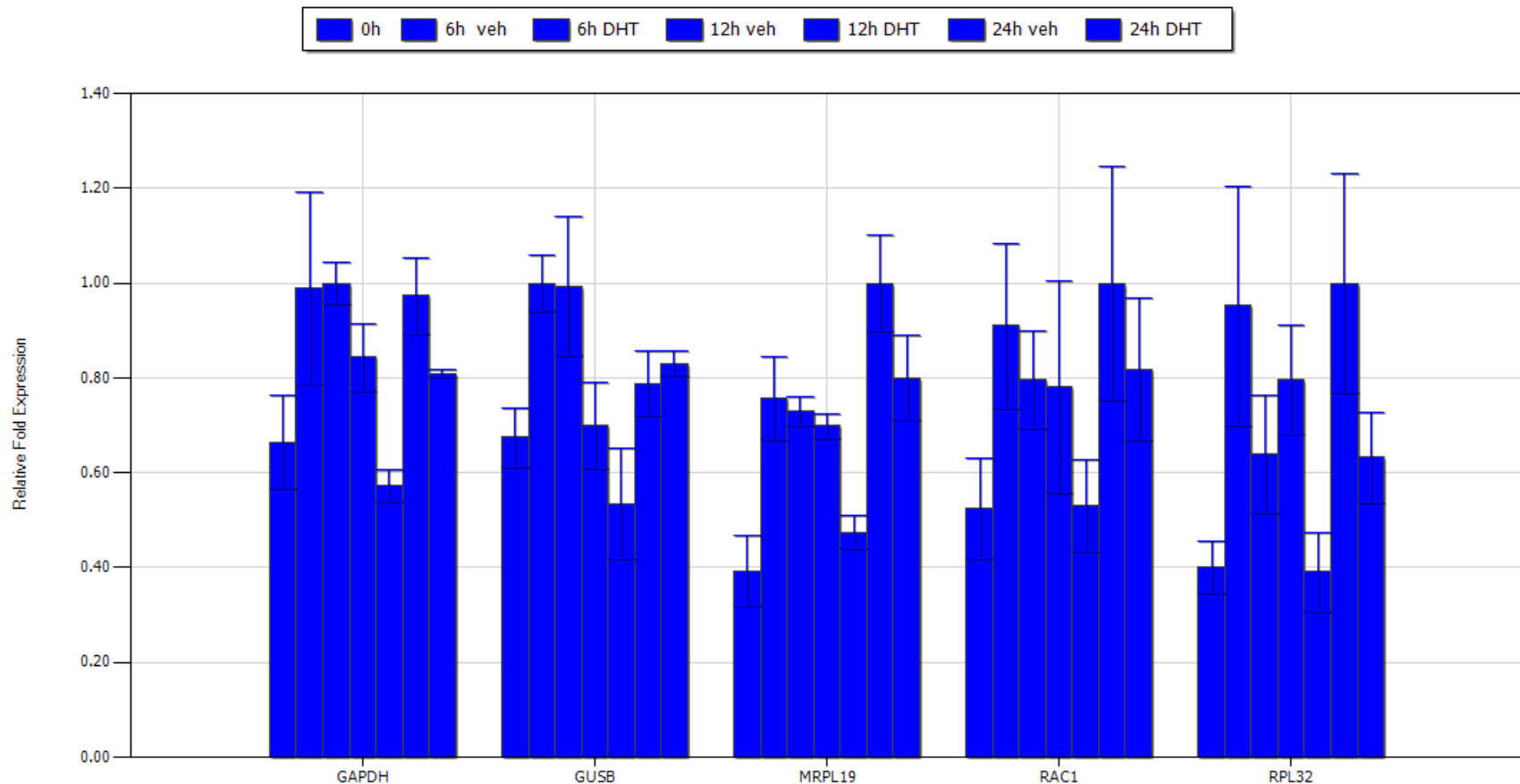
DHT

DHT + Bic

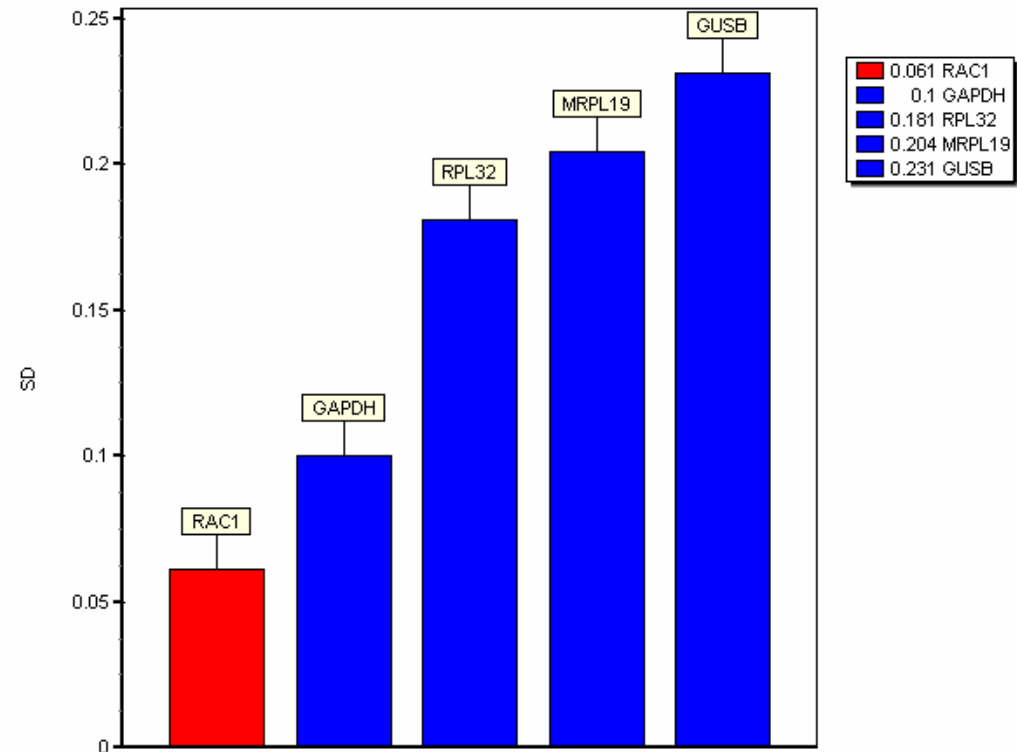
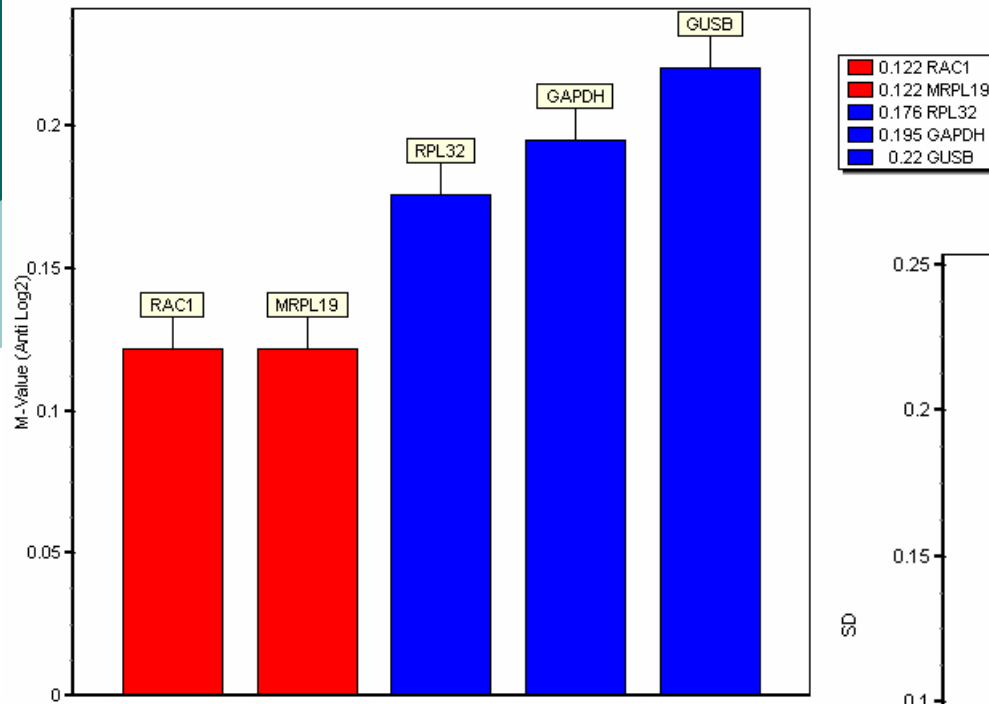


# RAC1 – novel reference gene?

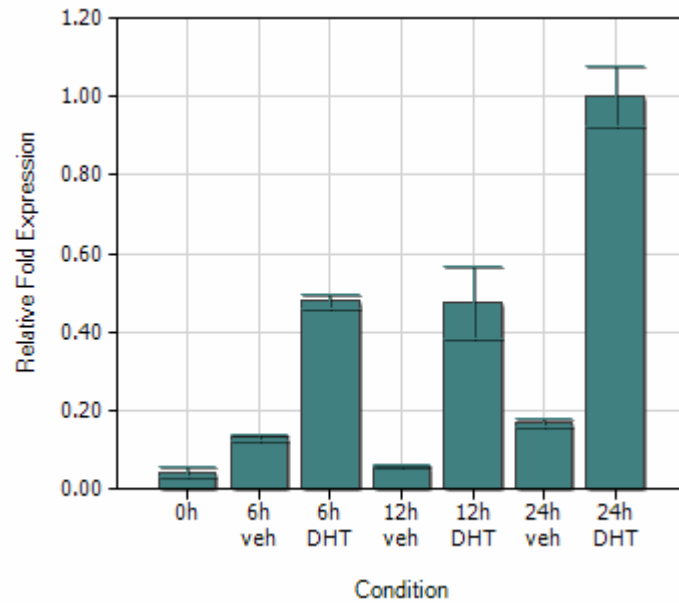
- When applied to LNCaP time course data, RAC1 still variable BUT.....



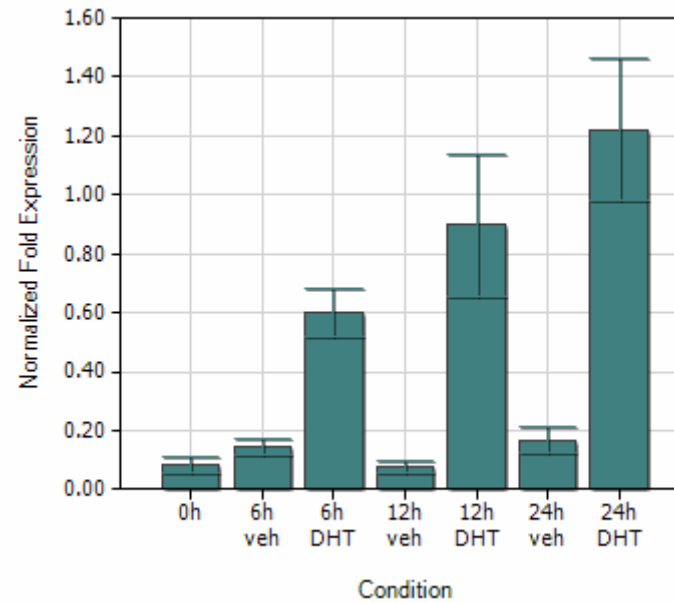
# geNorm and NormFinder



# KLK3 normalised to RAC1



**Non-normalised**



**Normalised to RAC1**



# Conclusion

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- **Hormone treatment alters RG expression**
  - Preliminary data looking at hormone receptor negative cell lines showed there was still variation - cell cycling effect?
  - Proliferation effects of hormones
  - From microarray data, there were very few reference gene candidates though novel way of identifying 'more stable' reference genes
- **Normalisation strategies**
  - May not be able to control for the effect of hormone
    - Quantitate to RNA input
    - Use of proliferation/cell cycling genes for normalisation eg BUB1, MKI67



# Acknowledgements

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