Real time RT-PCR normalisation: methods and considerations.

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Research projects at the Center for Infectious Diseases & International Health in the Developing World

- Clinical trials (Drugs, Vaccine, Diagnostic tests)
- Epidemiological studies
- Basic science research

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- **Basic science research**
  - **Immunology, Biochemistry, Microbiology, Genetics, Molecular biology**
- **Training/ Laboratory setup**
- **Troubleshooting, Support**
- **Assay development**

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Normalisation of real time RT-PCR

- Real time PCR can be very accurate over a large dynamic range
- Measuring RNA by RT-PCR accuracy can be effected by error:
  - Inherent variability of RNA
  - Multi-step process required for analysis
- Normalisation required to control for this error

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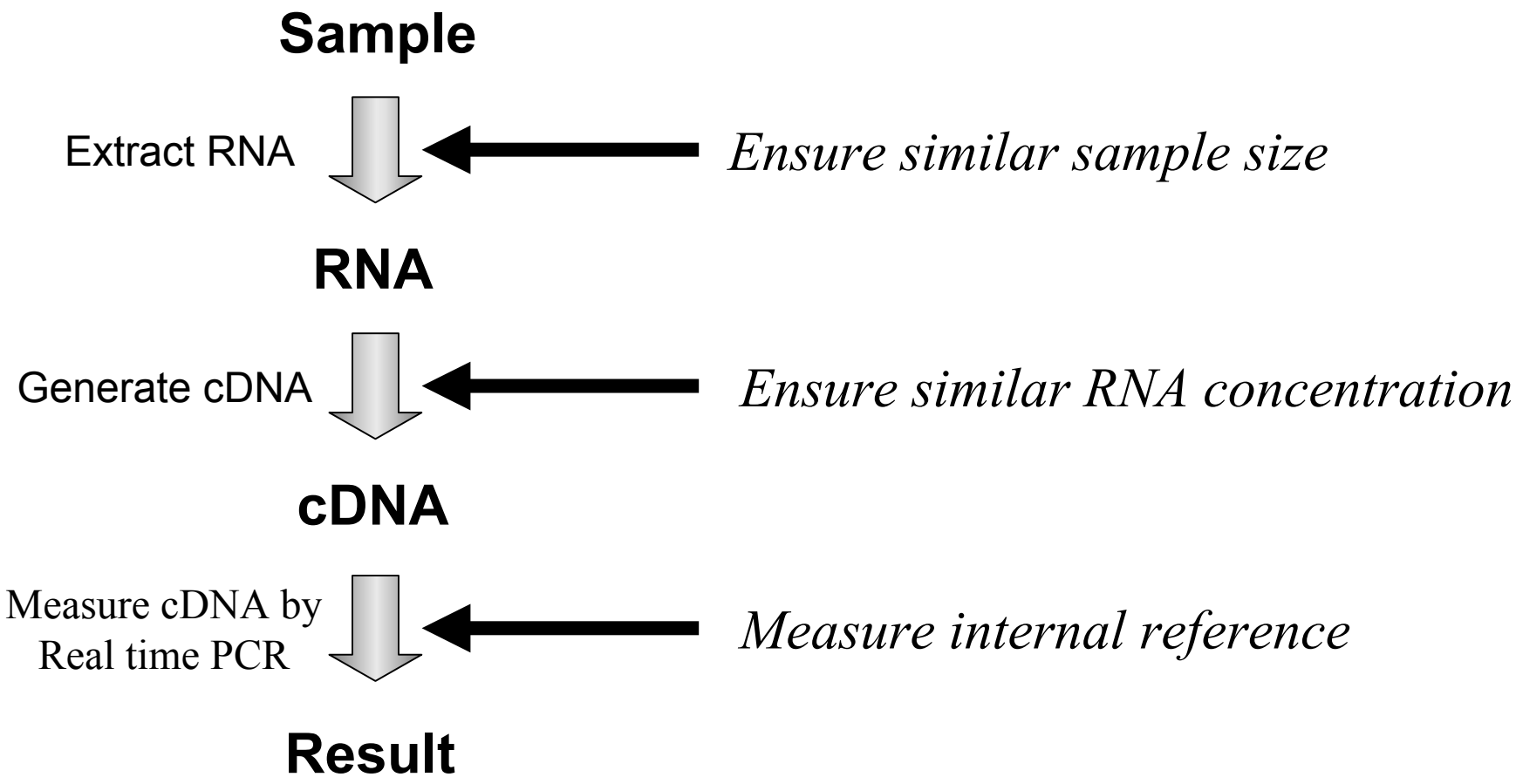
Normalisation of real time RT-PCR



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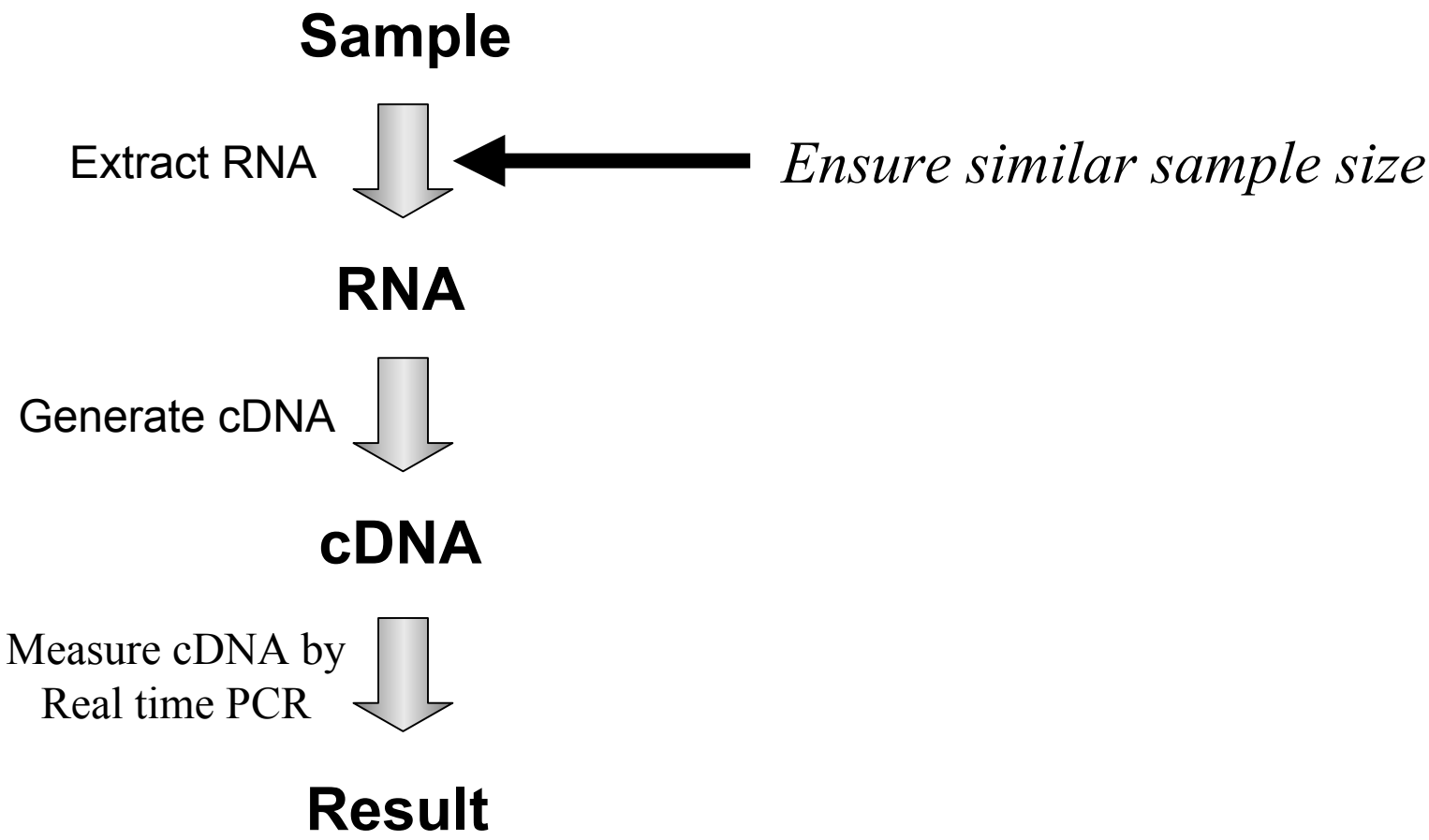

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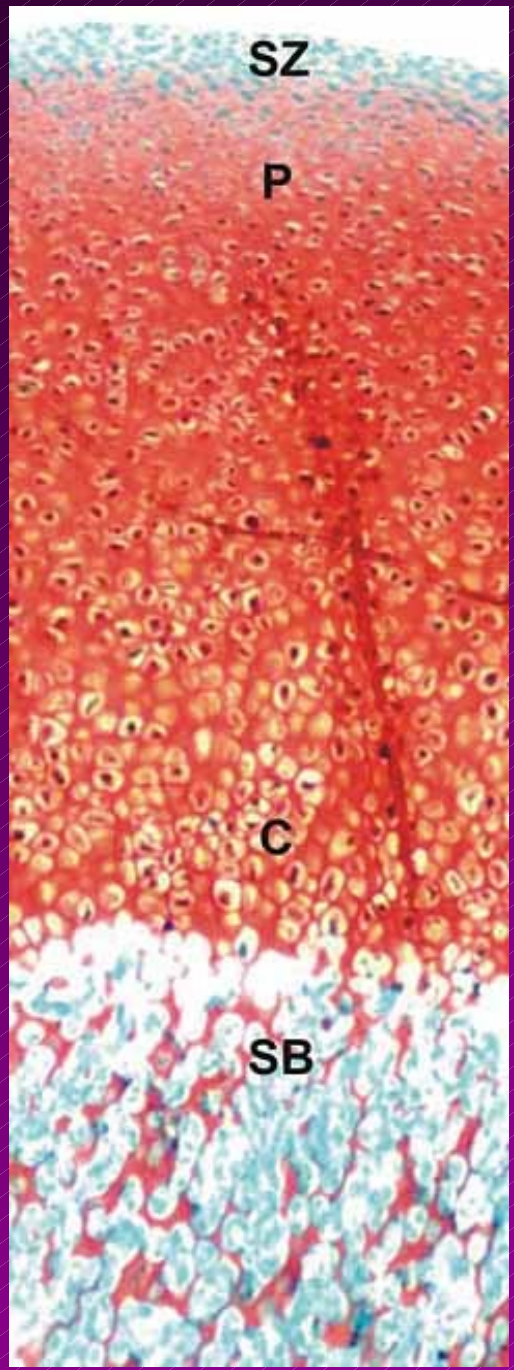

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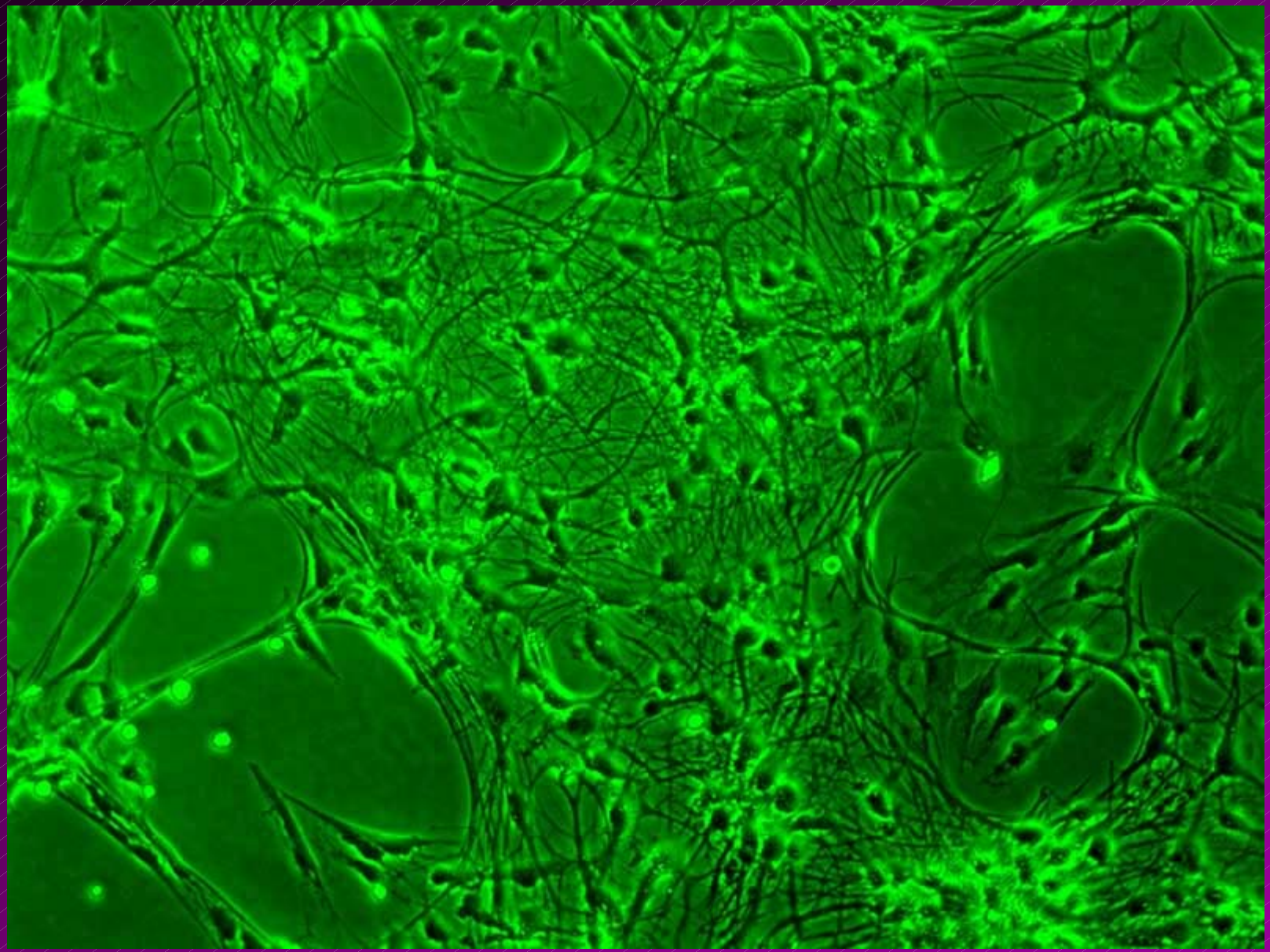

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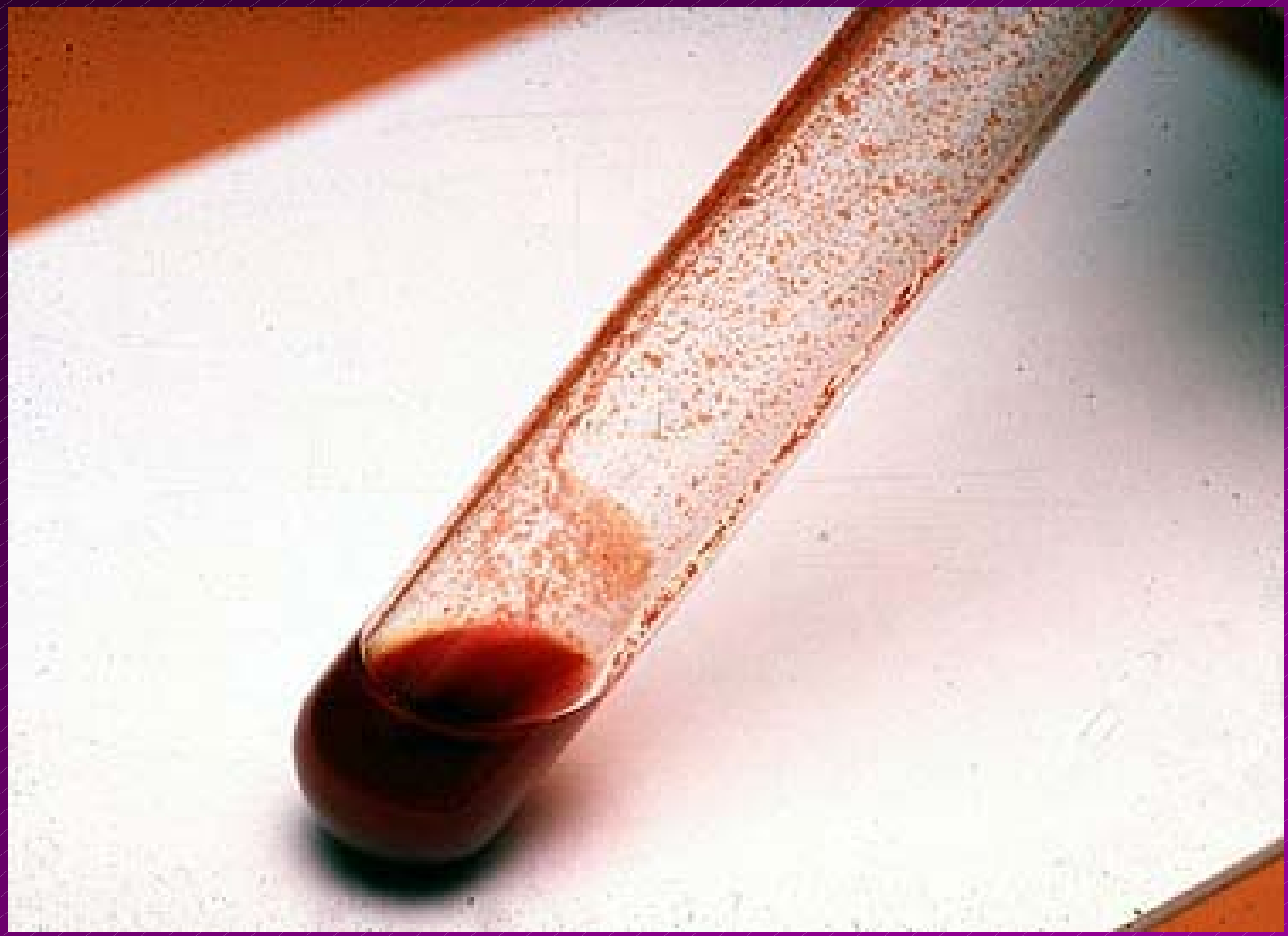

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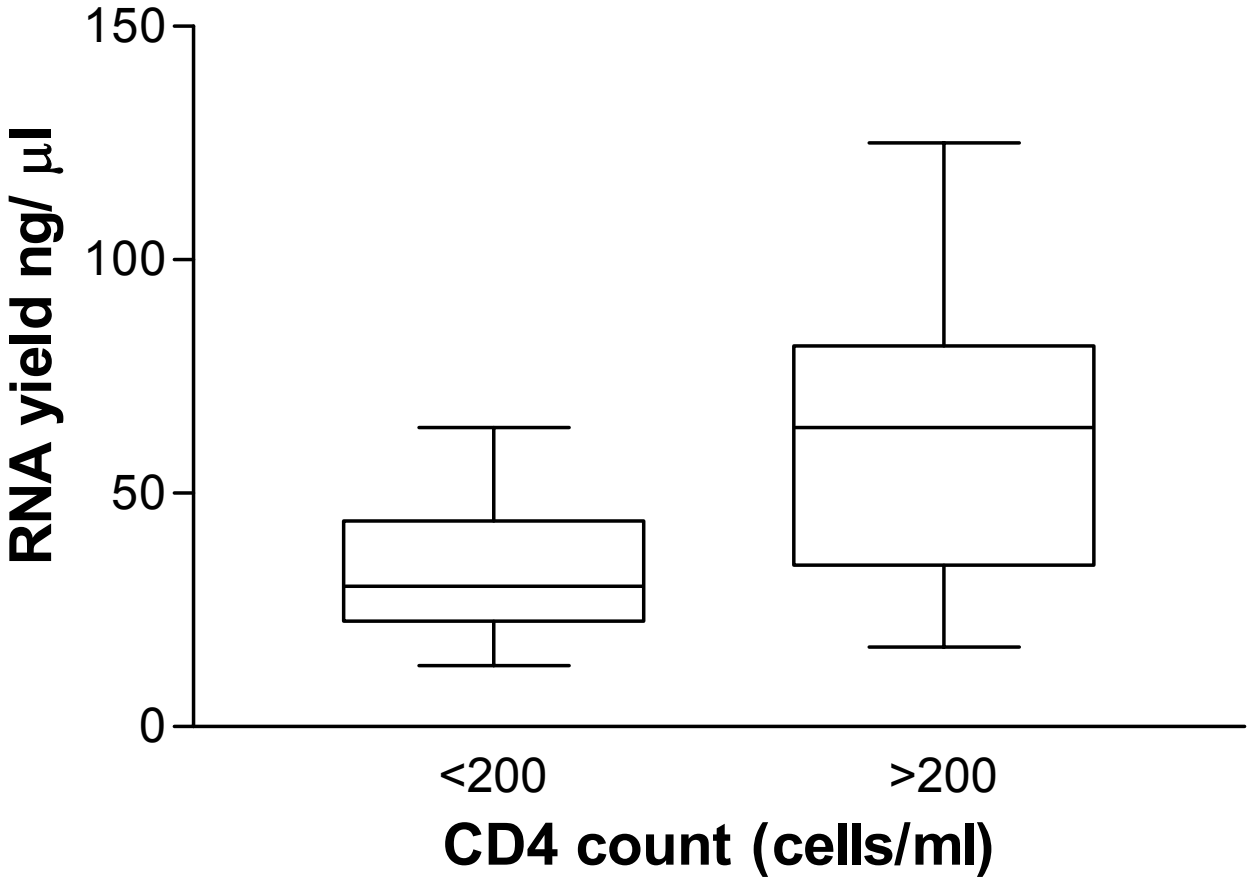
Total RNA extracted



p = 0.035

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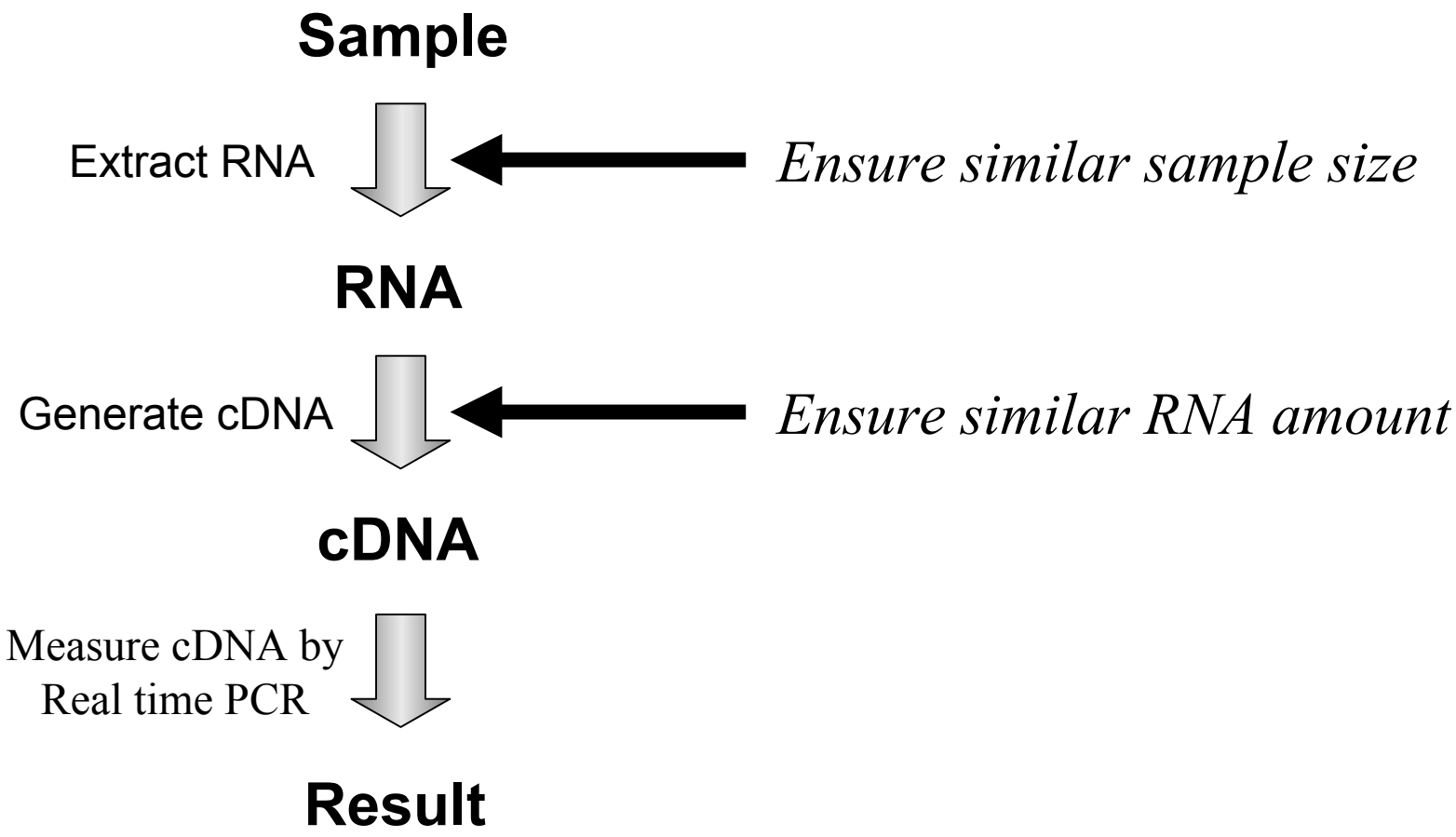

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Normalising to [RNA]

- UV (OD_{260})
- Gel analysis (Agarose, PAGE, Bioanalyser)
- Ribo-green

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Normalising to [RNA]

- Quantity
- Quality

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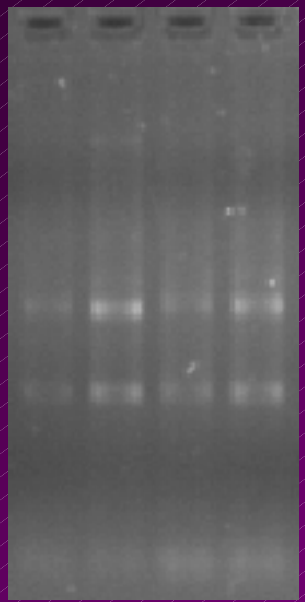
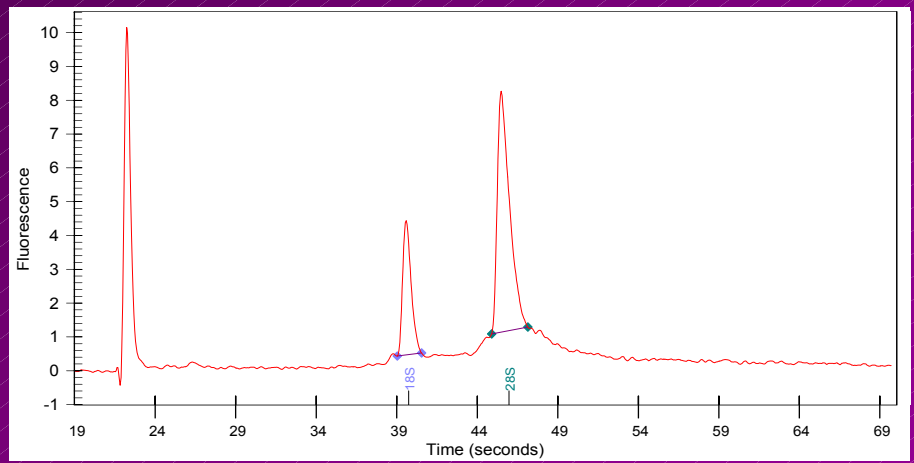
Normalising to [RNA]

- **Quality**





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Normalising to [RNA]

- Does not control for error introduced in subsequent steps
- Assumes no change in rRNA:mRNA ratio

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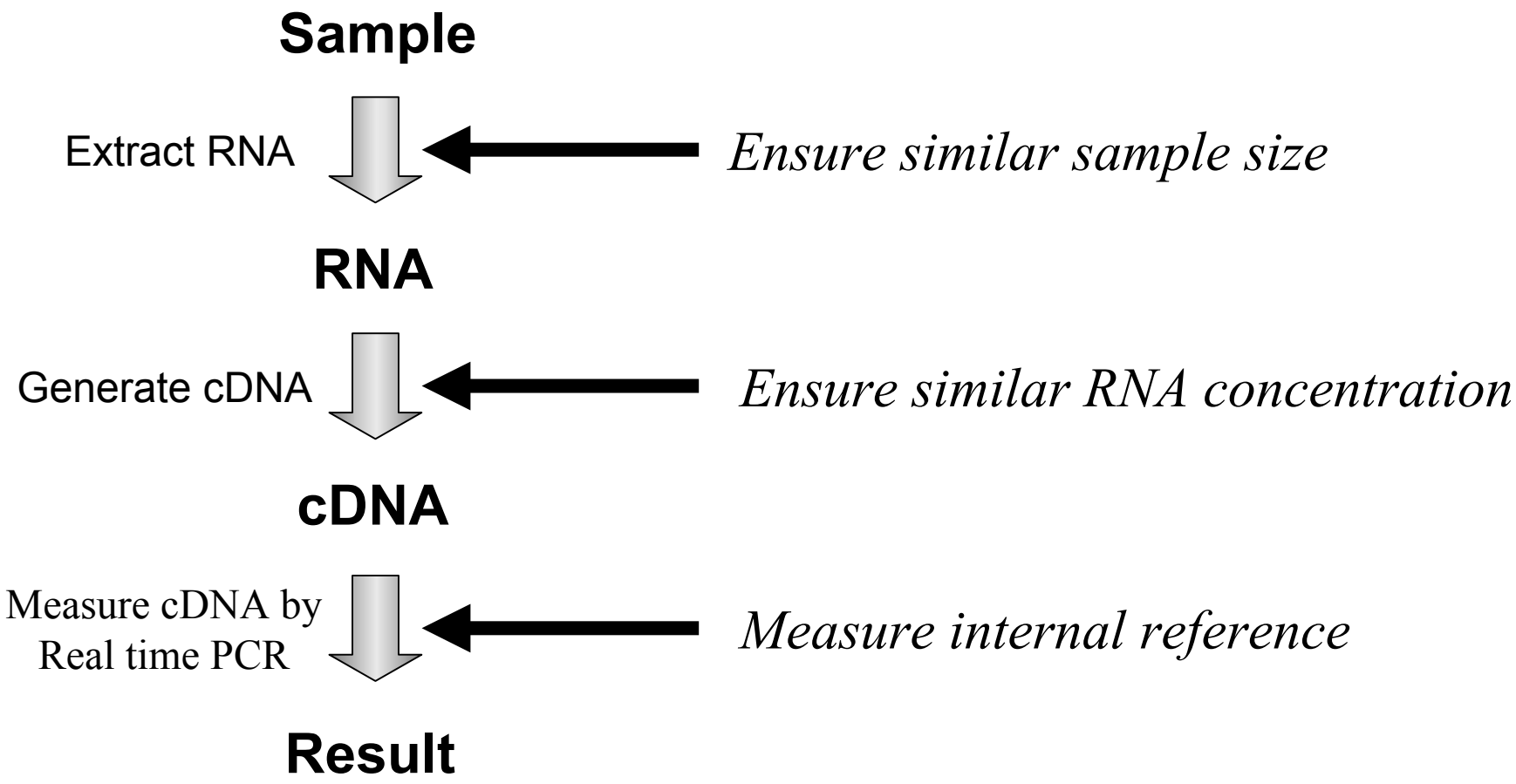

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Internal reference

- Reference Gene (House Keeping Gene)
- Artificial Spike
- Genomic DNA

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Reference genes

Genes that are assumed to be expressed at a constant level between experimental groups

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Reference genes

- Originate from RNA qualitative/semi-quantitative methods
- Examples:
  - glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
  - β-actin
  - hypoxanthine-guanine phosphoribosyl transferase (HPRT)
  - 18S rRNA
- Ubiquitous RNAs are ideal positive controls
- BUT are they constitutively expressed?

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Reference genes

- real time RT-PCR made RNA measurement quantitative over a huge dynamic range

- Surely when this happened the scientific world would have checked that reference were actually constitutively expressed.

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•Tanaka *et al.* 1975 18S rRNA was reported to increase in expression with cytomegalovirus infection.

•In 1984 Piechaczyk *et al.* reported that while GAPDH transcription occurred at a similar rate in different rat tissues they contained very different amounts of mRNA.

•In 1985 Stout *et al.* HPRT is constitutively expressed at low levels in most human tissues but was elevated in certain parts of the central nervous system

•In 1987 Blomberg *et al.* β-actin mRNA differentially expressed in different leukaemia patient tumour samples.

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- The notion that these RNAs require validation is also not new.

- 1989 Barbu *et al.* reported that probes to β-actin were inappropriate as a reference when comparing different mouse tissue by northern blot

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There is a lot of published data that has used invalidated reference genes for normalisation

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- Study investigating the immunology of Tuberculosis

- We used the Taqman Human Endogenous Control Plate to investigate 10 potential house keeping genes

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A.
Biotechniques. 2004 Jul;37(1):112-4, 116, 118-9.

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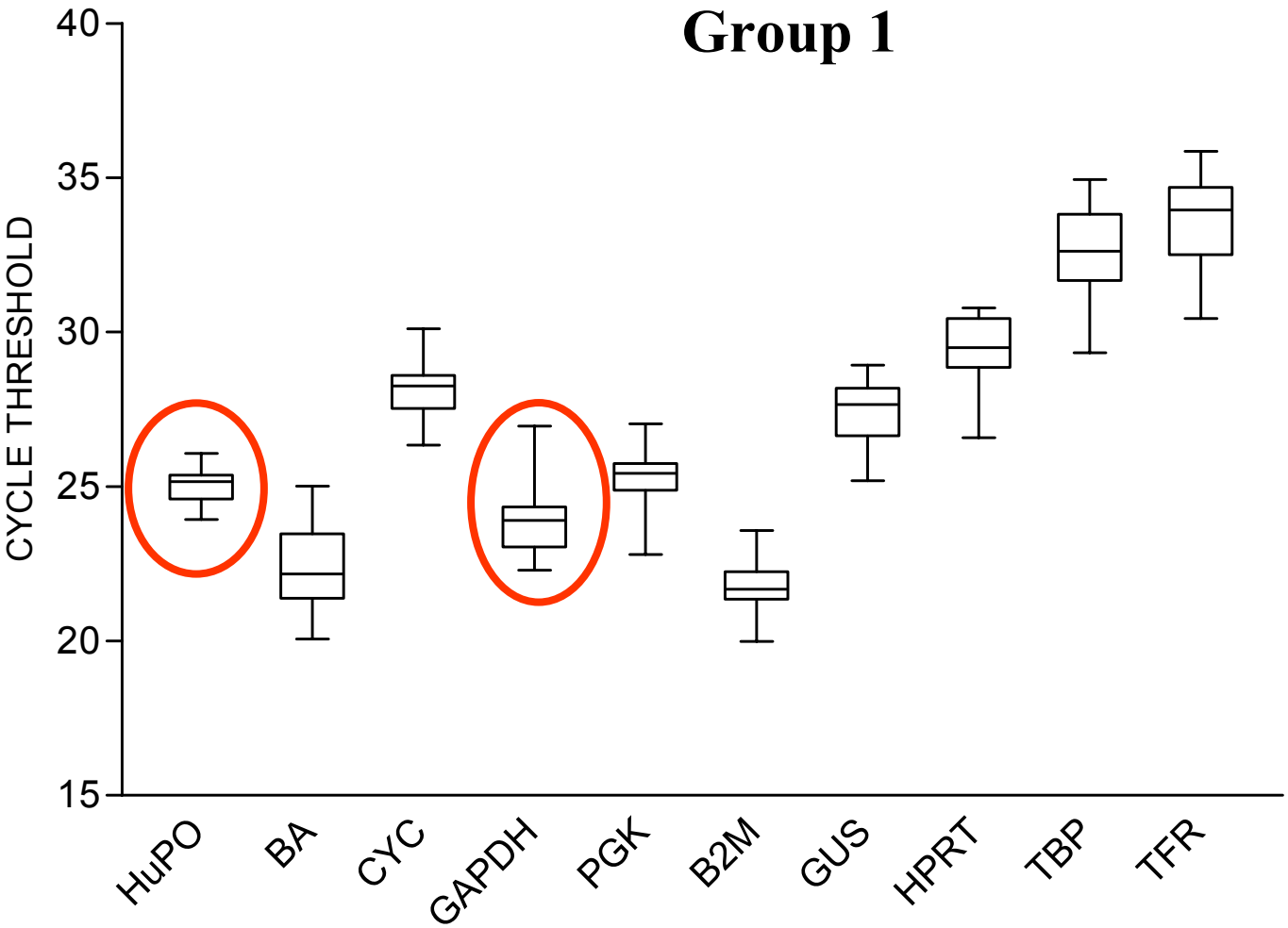
CT Variation of house keeping genes from human blood



Median, 25th & 75th percentile and range

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2 things to measure with reference gene validation

1. General error
2. Directional error (directional shift)

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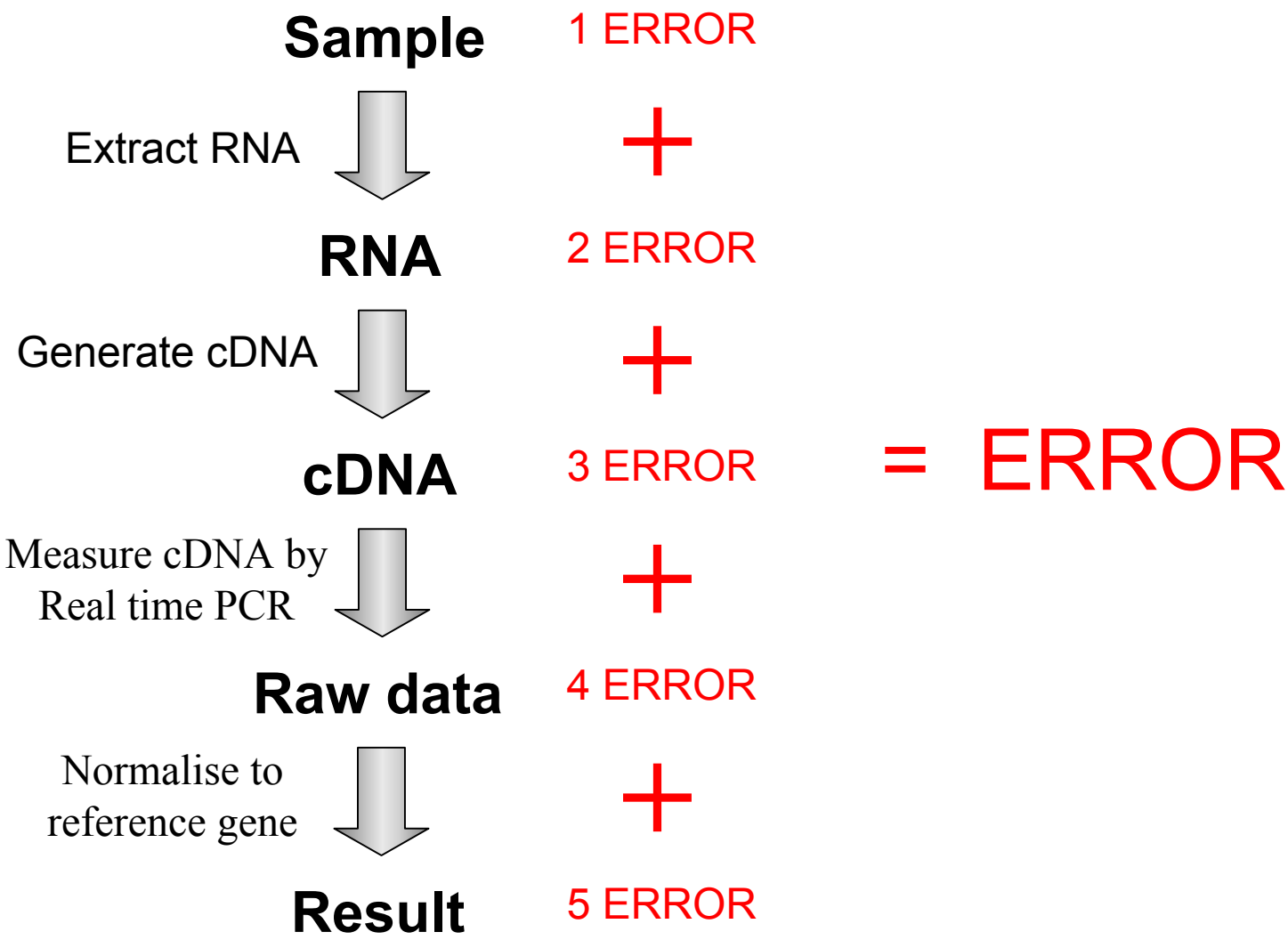
1. General error



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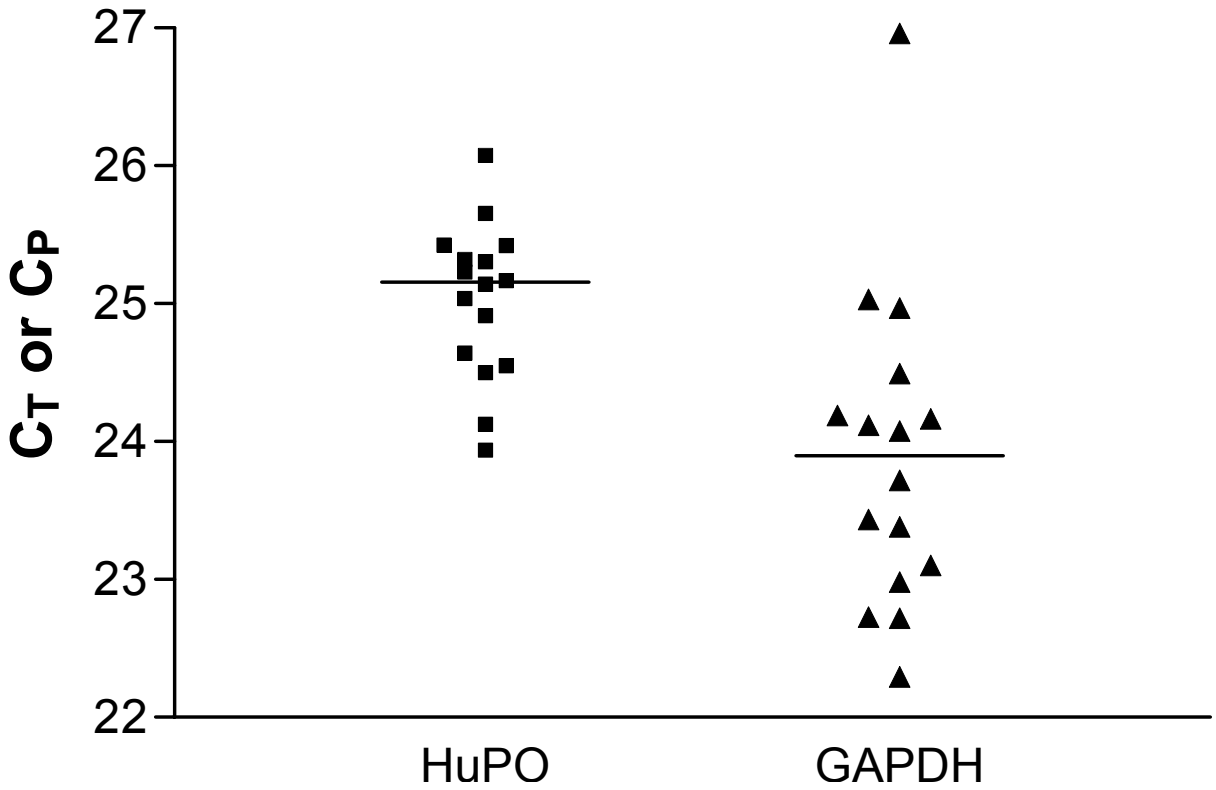
1. General error



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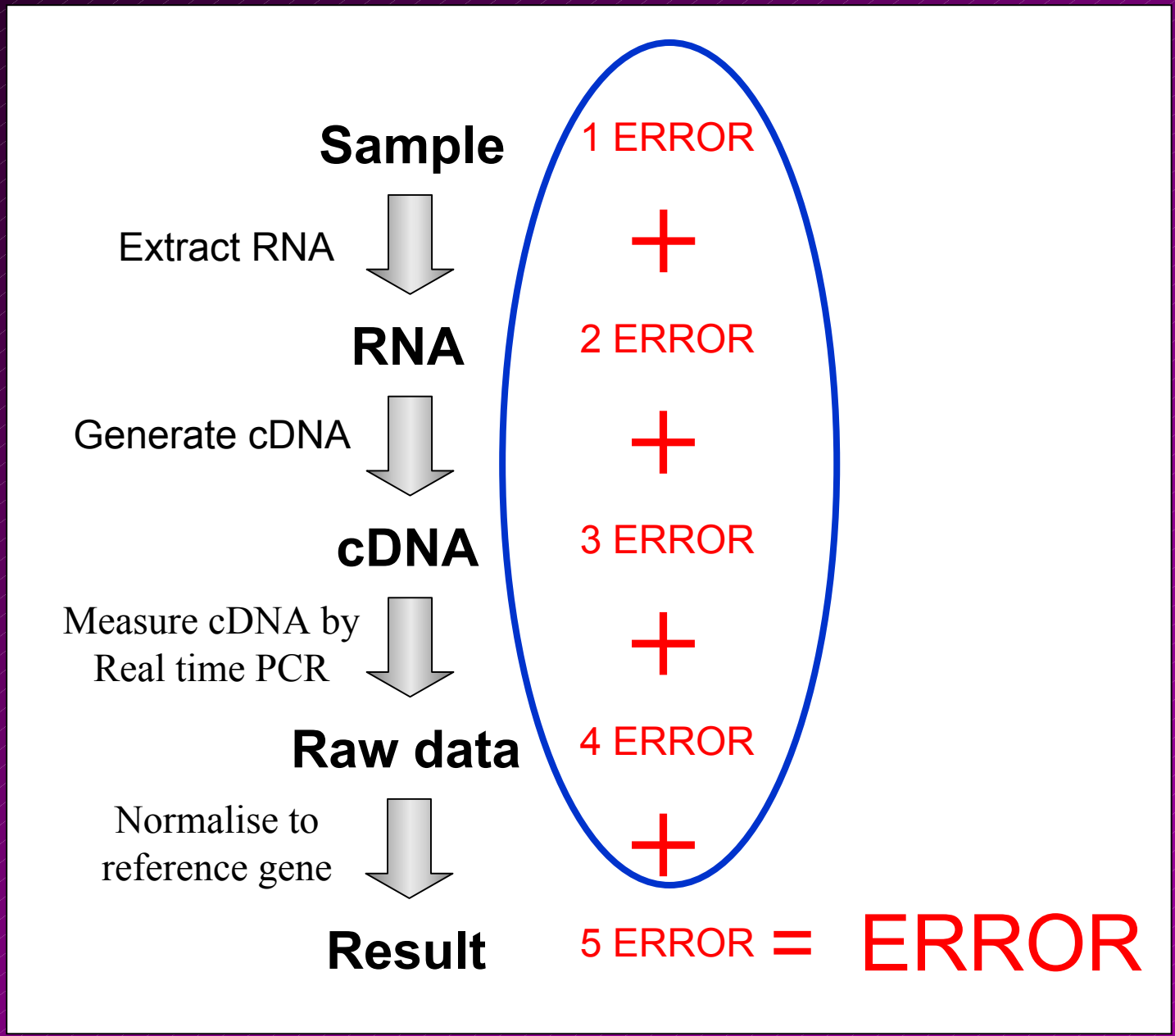
1. General error



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1. General error

- The error of the chosen reference gene defines the resolution of the assay

- This resolution is dependent on the desired measurement

  i.e. 1 log variation in reference gene is OK for a 2 log measurement

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2. Directional Shift

- Assumption reference gene constitutive expression
- Variation reference gene expression is always random
- What if the experiment effects chosen reference?

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2. Directional shift effect: example 1

- Comparison of Interleukin 4 (IL4) mRNA expression between Tuberculosis patients and healthy individuals.
- Normalise raw data to HuPO or GAPDH

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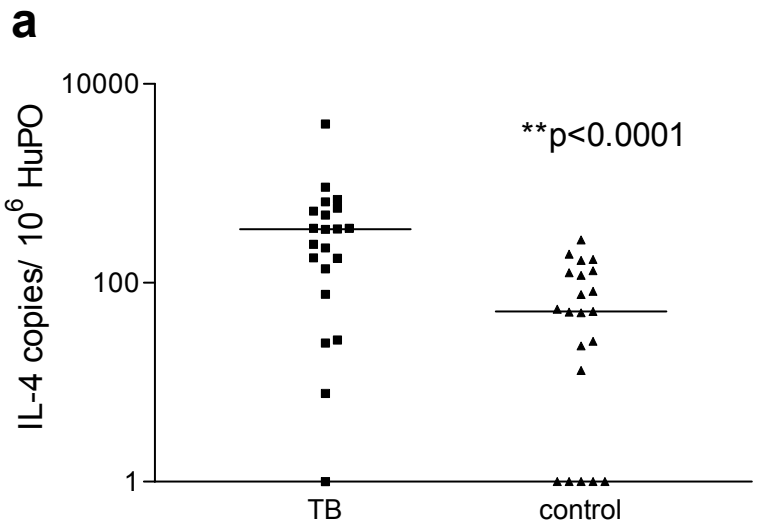
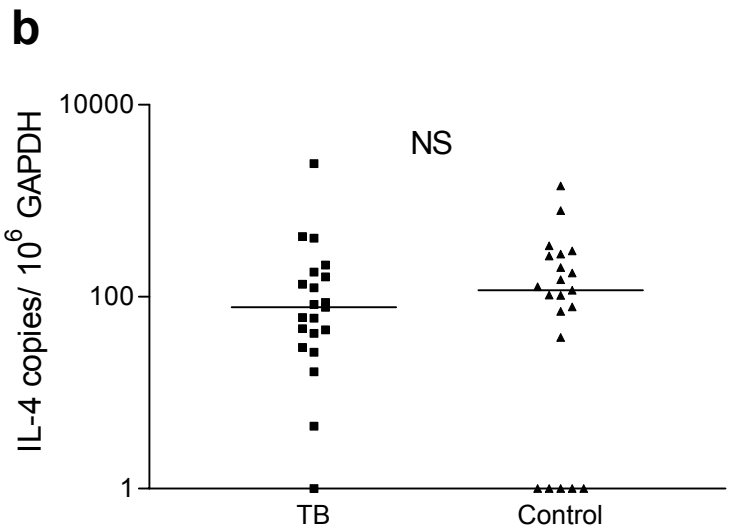
2. Directional shift



Normalised to 10^6 copies HuPO

Normalised to 10^6 copies GAPDH



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2. Directional shift effect: example 2

- Effect of treatment on IL4 mRNA expression
- Normalise raw data to HuPO or GAPDH

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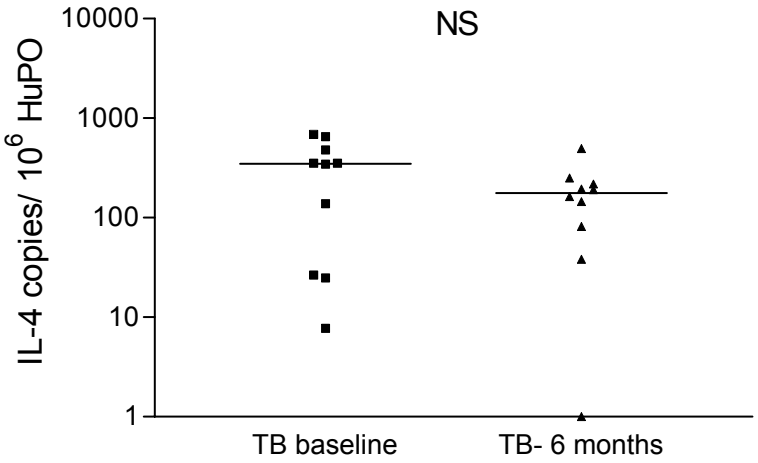
2. Directional shift



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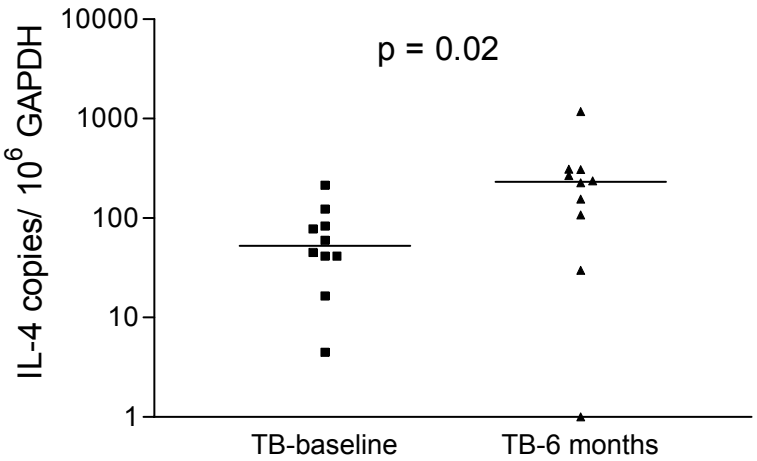

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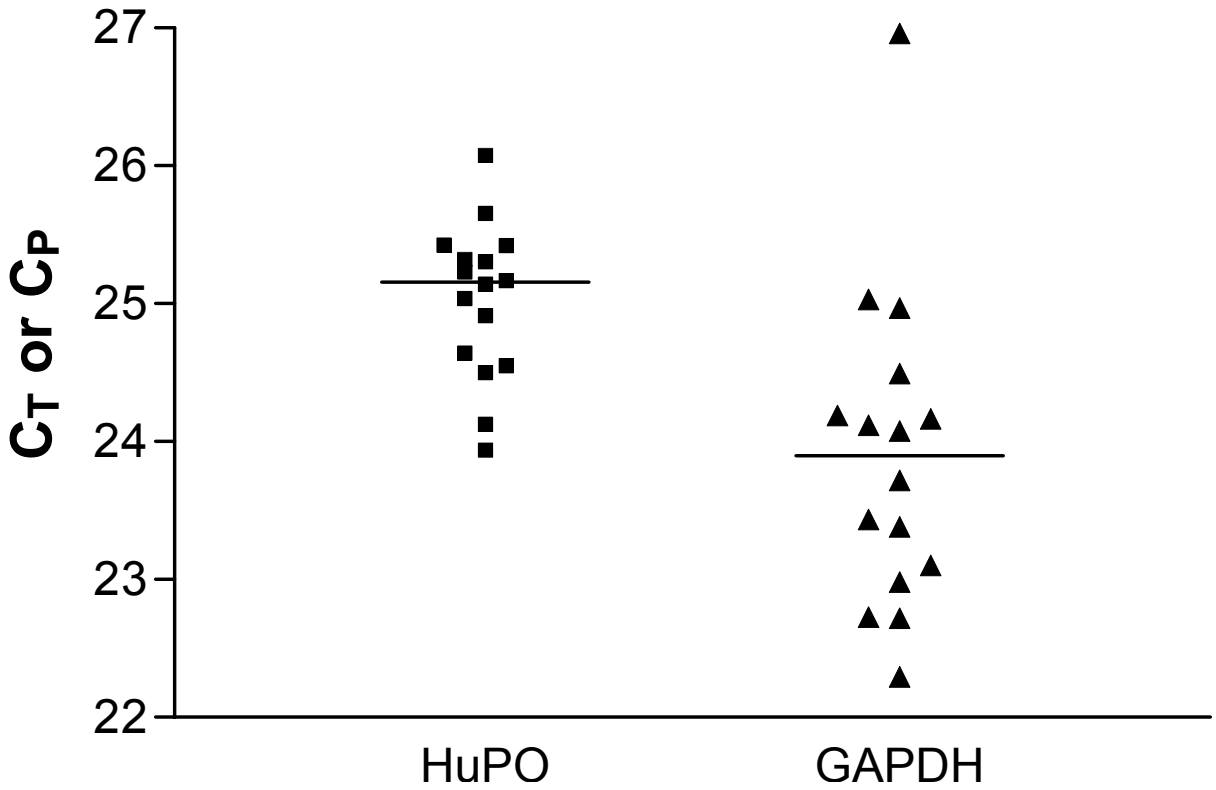
1. General error



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Conclusion

- There are a number of methods for normalising real time RT-PCR experiments which are not mutually exclusive
- Using a reference gene is a simple and effective method
- However it must be validated for the specific experiment using the actual samples being measured
- Failure to do so may generate incorrect results

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Acknowledgements

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