Using digital PCR to characterise viral vector genomes

Dr Alexandra Whale, Science Leader, Nucleic Acid Innovation
National Measurement Laboratory, LGC, UK

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National Measurement Laboratory (NML)

Measurement matters

Overview

- National Measurement Laboratory
- Viral vectors and gene therapy
- Process of digital PCR (dPCR) and how it quantifies nucleic acids
- Where can dPCR support characterisation of viral vectors
- Specific example using dPCR to quantify AAV viral vectors

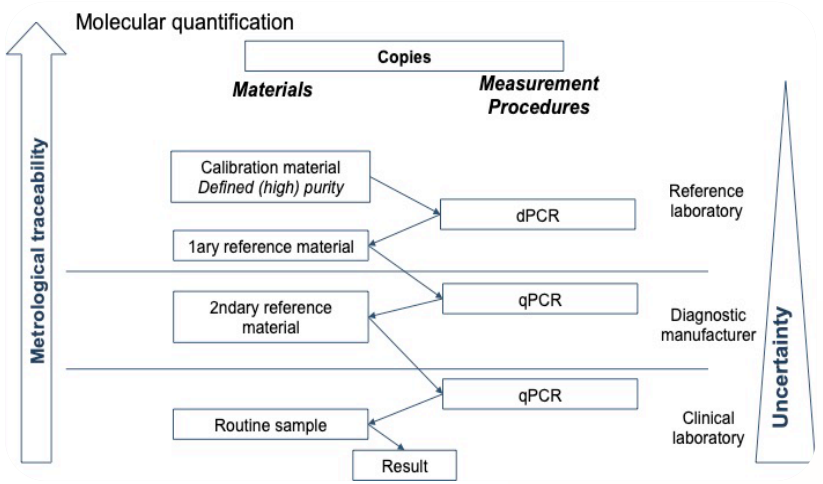
The Molecular Biology Laboratory

LGC, Queens Road, Teddington, TW11 0LY, United Kingdom

What is Metrology?

- **Metrology is the science of measurement**
- **It allows us to**
  - understand sources of error
  - enable traceability
  - improve measurement accuracy
- **We conduct measurement research to improve nucleic acid analysis**

ISO17511

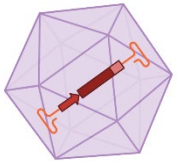


Viral vectors and gene therapy

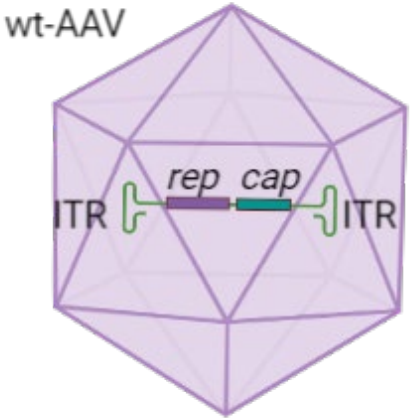
- **Gene therapy**
  - To treat genetic diseases by restoring defective gene functions
  - Adding functional genetic material to produce the missing protein or replace the defective protein
  - E.g. transduction of patient T-cells with lentiviral vectors to generate CAR-T cells to treat cancer
- **Biomanufacturing**
  - To modify a wide range of cells and tissues to produce therapeutic products
  - E.g. vaccine production

- **Viral vectors are modified viruses**
  - Efficient tools for gene delivery - naturally evolved to deliver their nucleic acid to infected cells
  - Encode a recombinant gene in their genome to produce a gene therapy product

Common viral vectors
Adenovirus
Adeno-associated virus (AAV)
Retrovirus/Lentivirus
Poxvirus
Herpes-simplex virus
Baculovirus

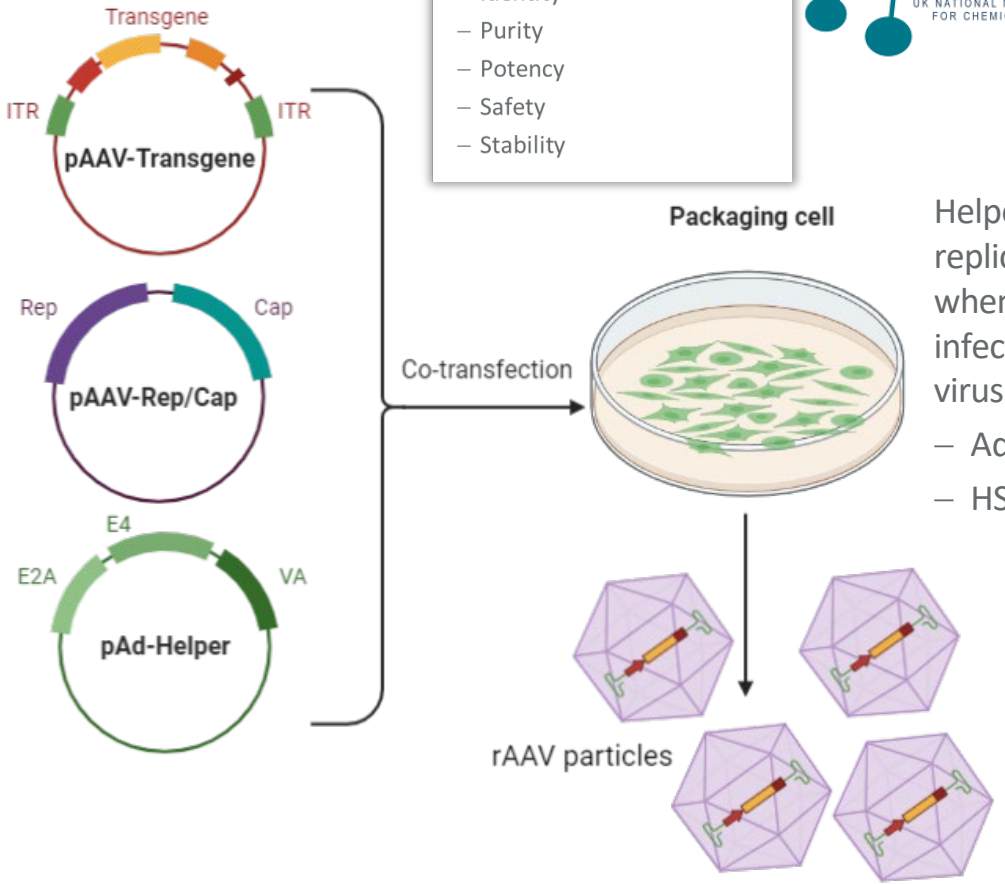


AAV viral vector introduction



Adeno-associated viruses (AAV) belong to the *Parvoviridae* family

- small (~20nm) non-pathogenic, non-enveloped, icosahedral viruses
- Genome: 4.8 kb single-stranded DNA



Critical Quality Attributes (CQAs)

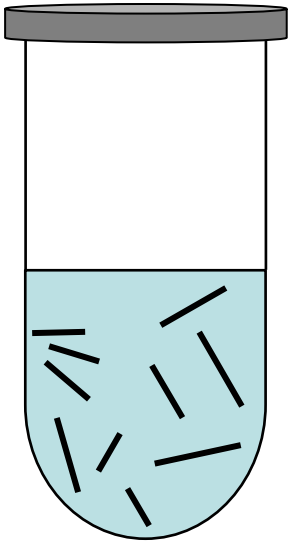
- Identity
- Purity
- Potency
- Safety
- Stability

Helper-dependent: replication only when cell is co-infected with helper virus

- Adenovirus
- HSV-1

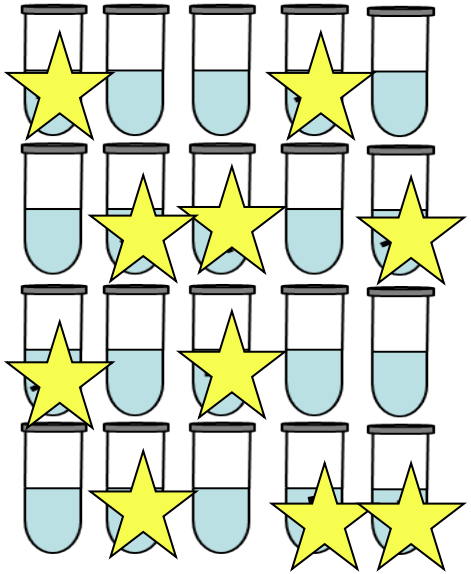
What is digital PCR?

Real-time PCR
1 × 20 µL reaction



Split sample by dilution

Digital PCR
20 × 1 µL reactions



positive = 10 total = 20

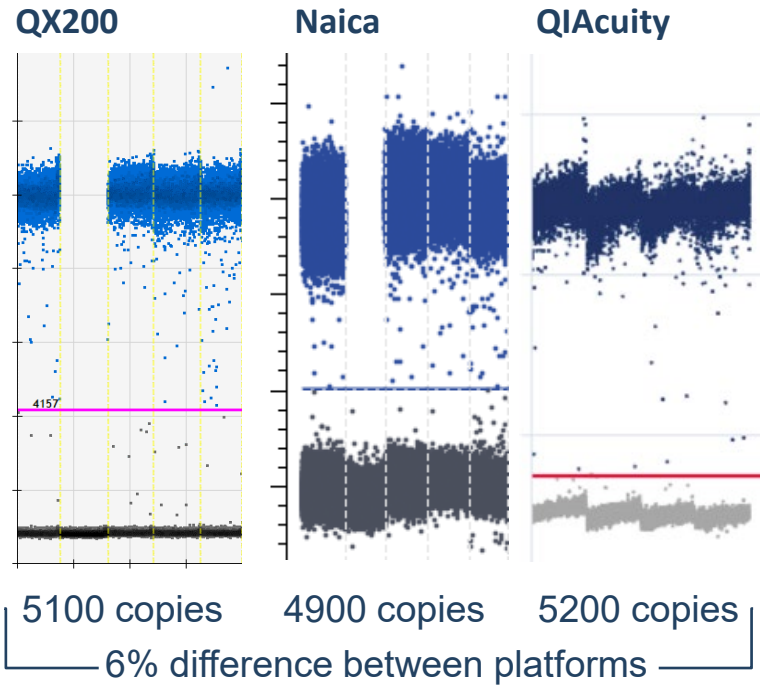
Prepare a real-time PCR reaction

- Enzymes, buffers, dNTPs etc
- Primers (& probes)
- Nucleic acid template

- **Subdivide into discrete partitions**
  - Limiting dilution; some partitions contain no template
- **qPCR performed as normal**
- **Read each partition**
  - Assigned as positive or negative
  - Proportion of positive partitions is used to estimate the number of template molecules

How can dPCR support viral vector characterisation?

- **Absolute quantification with no calibration curve to interpret the data**
  - Counting (proportion of positive partitions out of the total) based on nucleotide sequence
  - Predictable precision and reproducible measurements
  - SI traceability we can use the dimensionless quantity taken to have the SI unit “one”
- **Bespoke nature of viral vectors make it tricky to develop reference materials**
  - pTransfer vector contains the vector genome sequence
  - Correct viral genome packaged versus not correct
  - Use the partitioning of dPCR to investigate linkage of targets



How can dPCR support viral vector characterisation?

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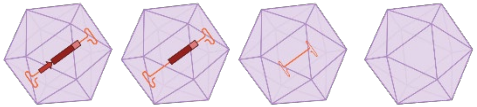
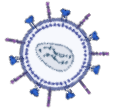
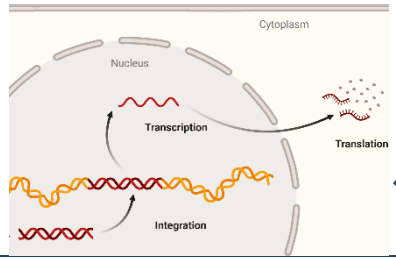
Quantity: Viral titre/mL
Quality: % correct genome



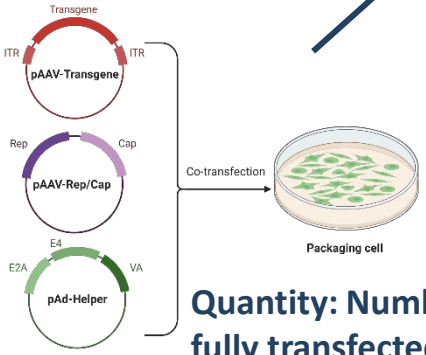
Quality: Integration efficiency and stability



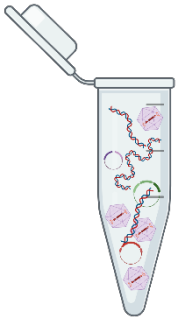


Quantity: Expression of the gene therapy transcript

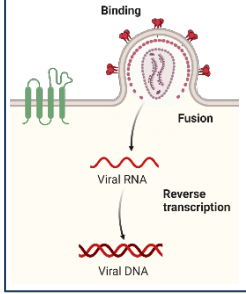
Viral vector selection and design → Transfection of packaging cell → Production of viral vector in packaging cell → Viral particle purification → Transduction of host cell with viral particles → Characterise host cells



Quantity: Number of fully transfected cells



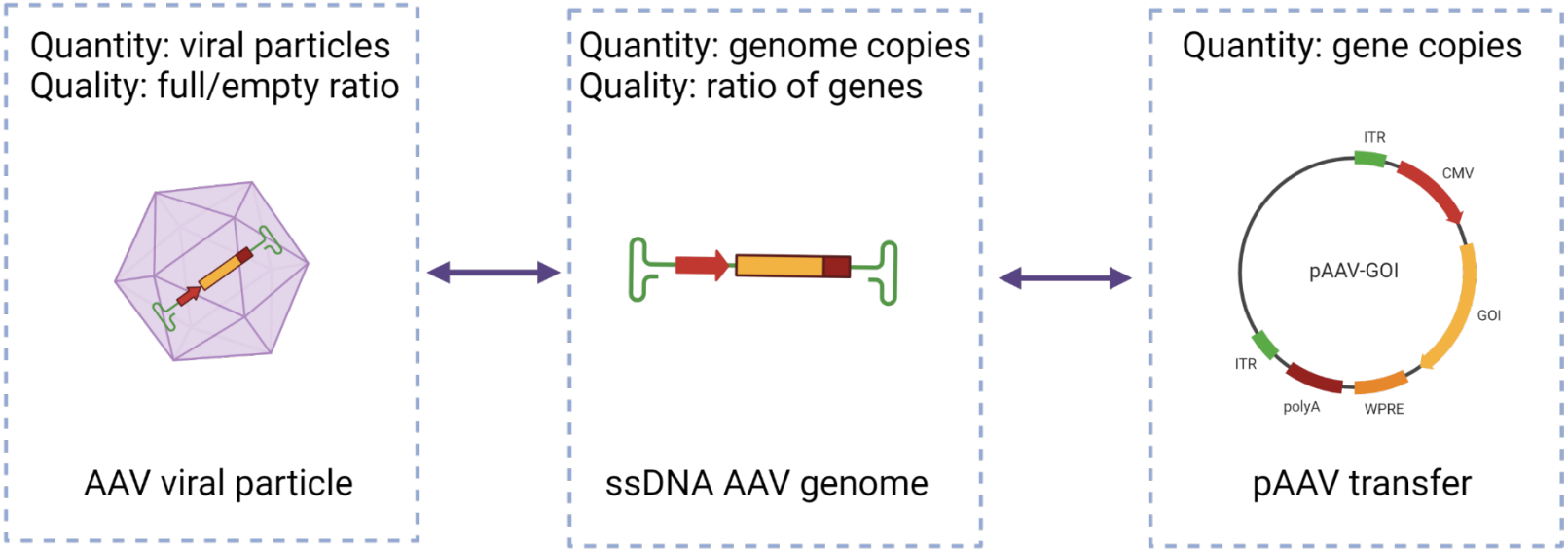
Purity: Packaging cell contamination
Purity: Transfer or Helper plasmid remnants



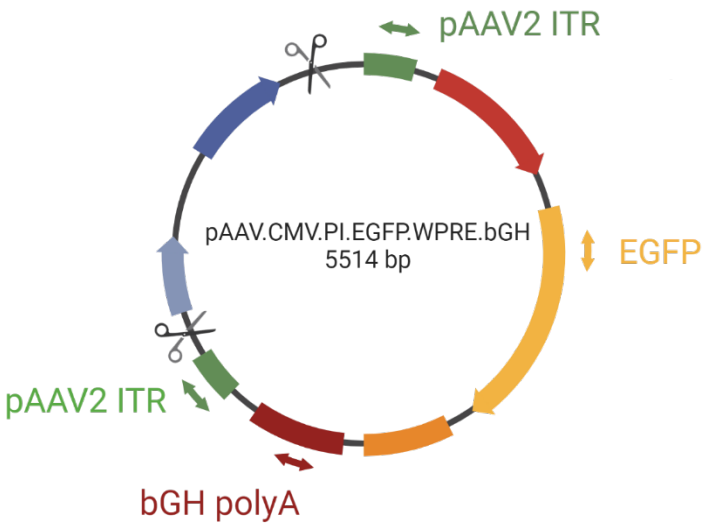
Quantity: Gene delivery into the host cell

LGC

Workflow for AAV vector genome characterisation using dPCR

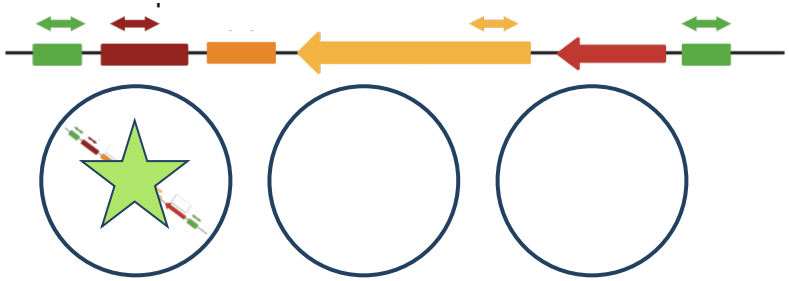


Digital PCR method development



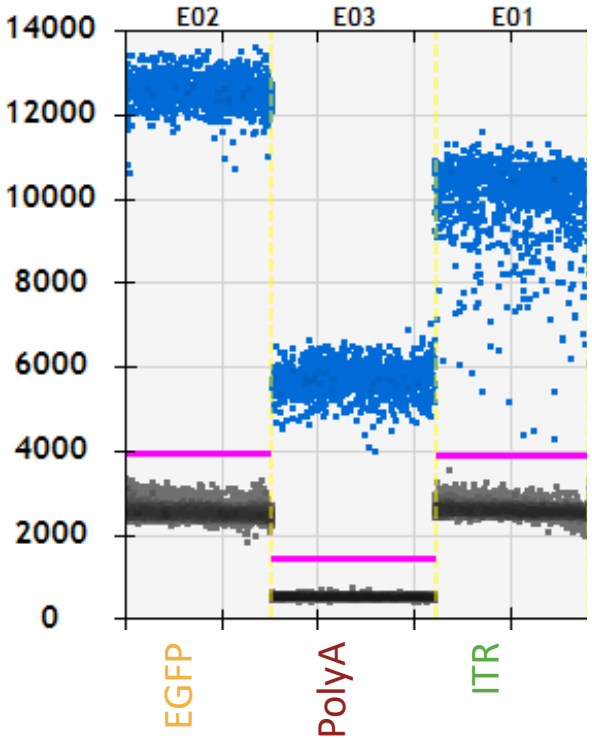
- **Use a pAAV2 transfer vector to develop and validate the dPCR method**
  - Encodes EGFP as the gene of interest
  - Restriction digest of pAAV2 transfer vector
  - Surrogate for AAV2 genome
- **Ratio of different targets across the genome to determine full or partial genome**
  - One target per genome: EGFP and bGH PolyA
  - Two targets per genome: ITRs (palindromic)

Digital PCR method development



– Separate reactions per target

BioRad QX200



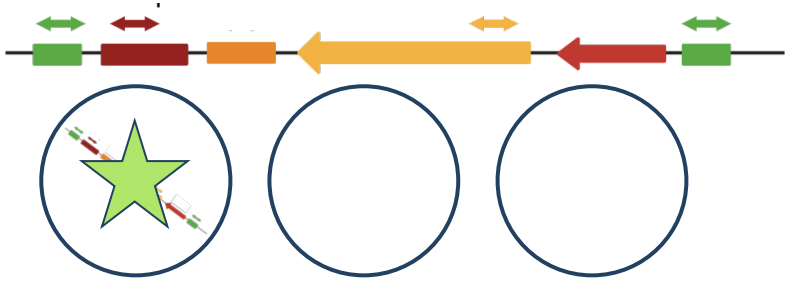
Assay optimisation:

54-64 °C annealing temp gradient (58 °C shown)

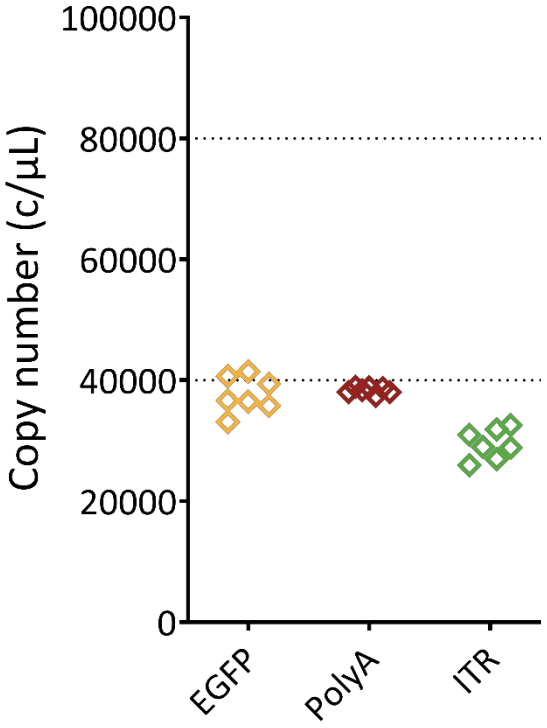
Match/mismatch

Dynamic range (~100,000 to 100 copies/rxn)

Digital PCR method development



- Separate reactions per target
- Good agreement between the single copy targets



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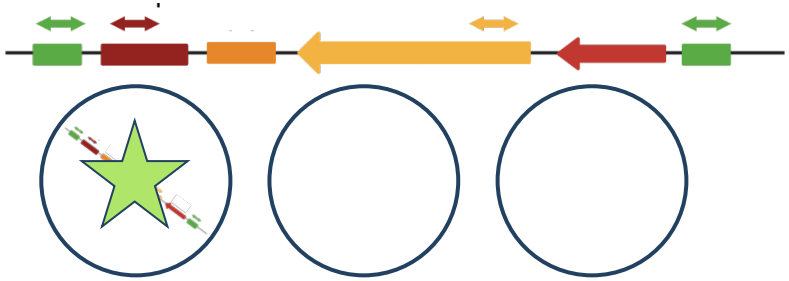

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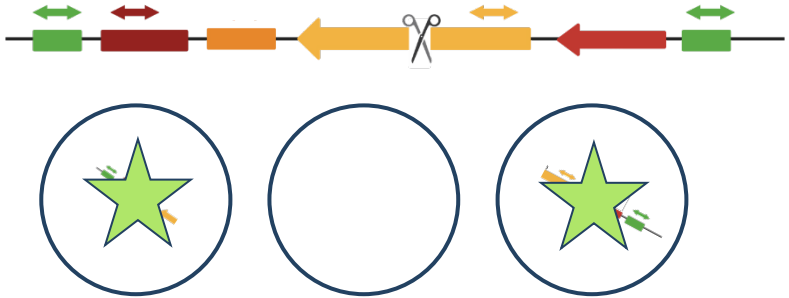
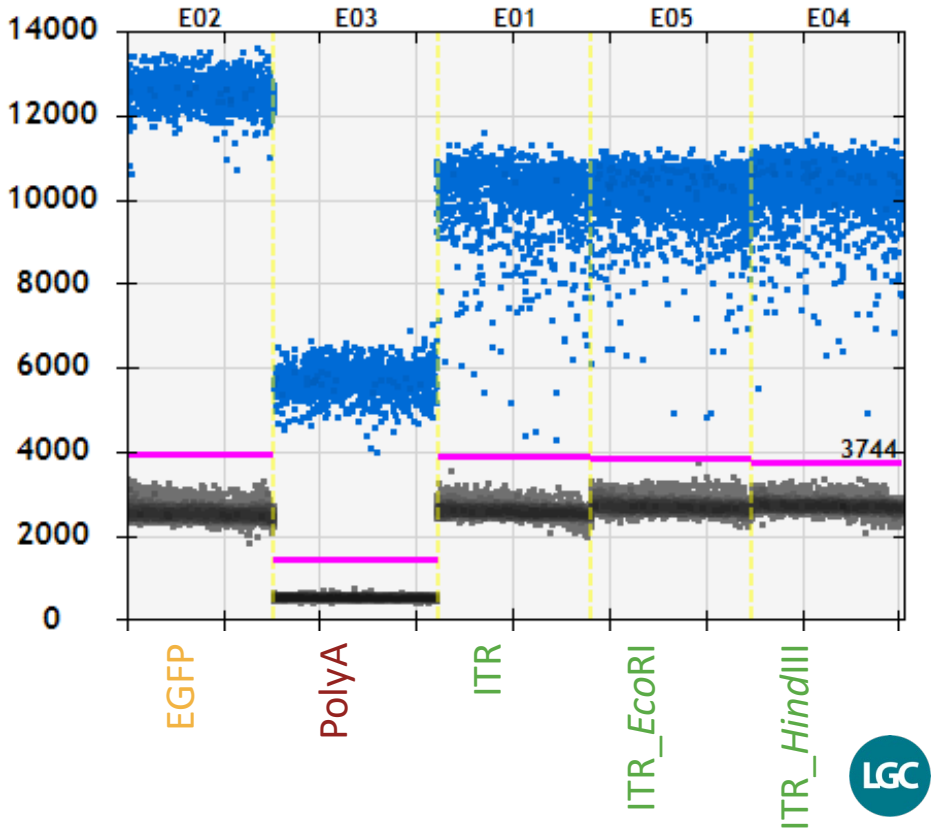


- ITR separated using restriction digestion

BioRad QX200



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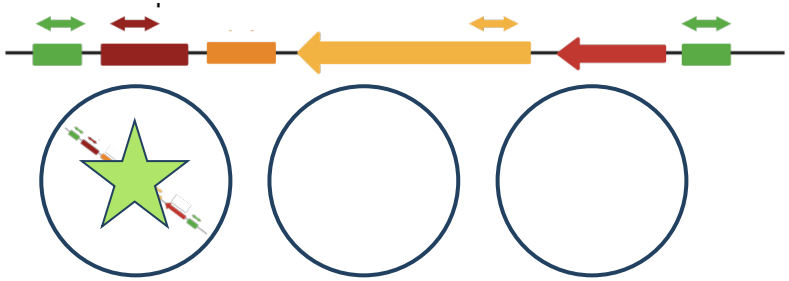

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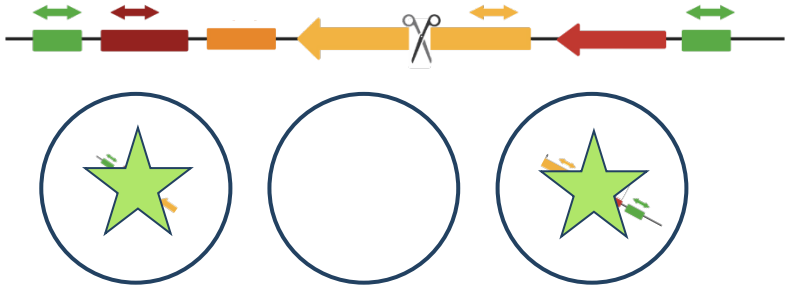
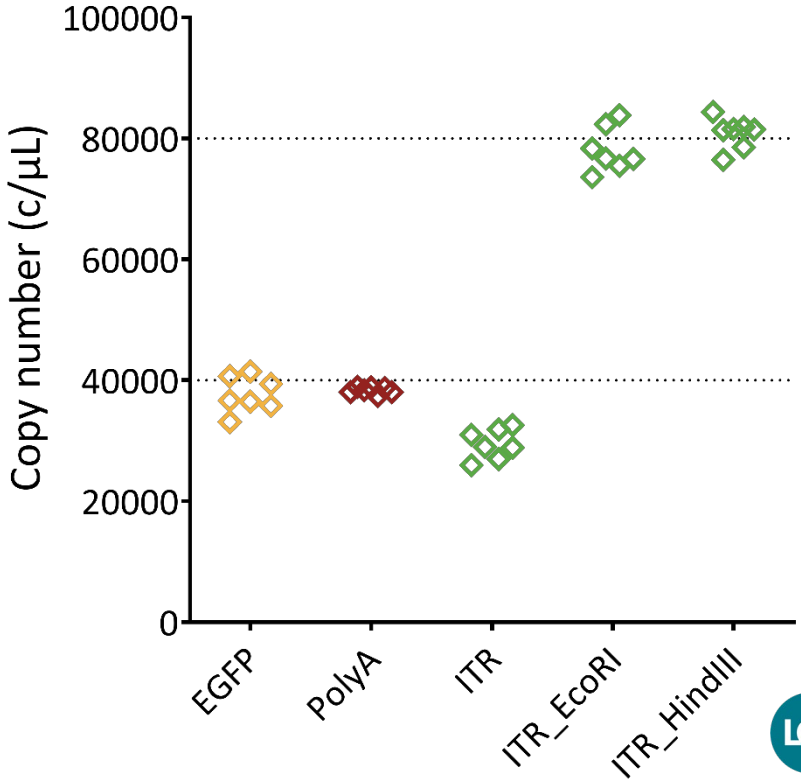
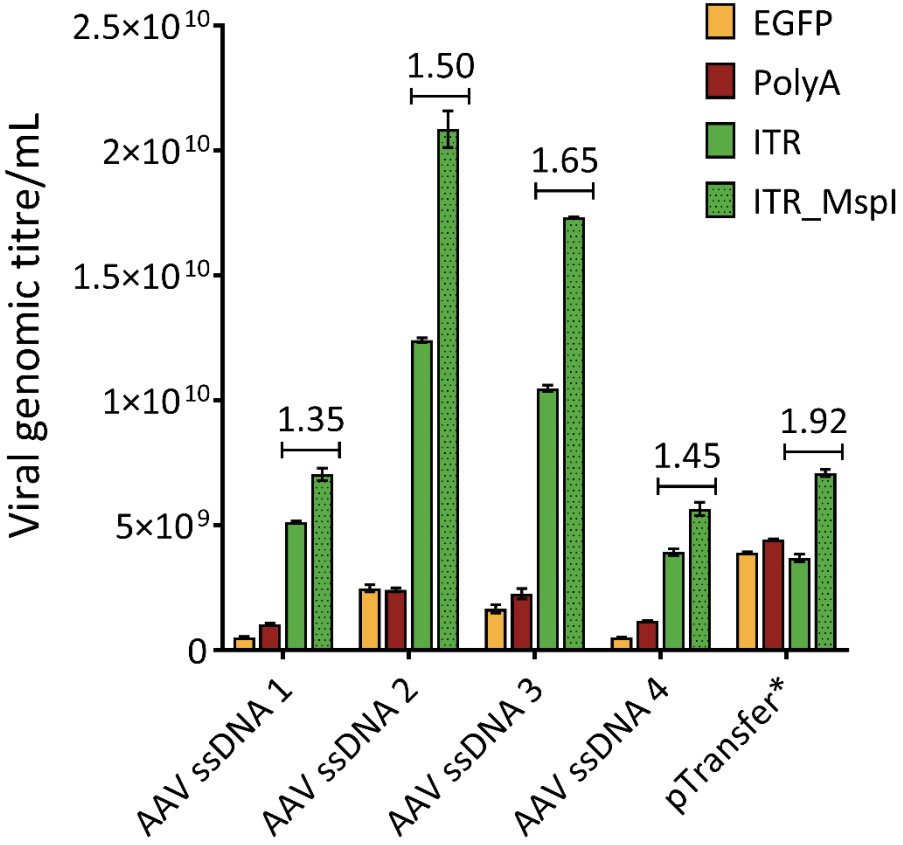


- ITR separated using restriction digestion
- Digestion doubles number of ITR copies measured by dPCR



Quantification of AAV genome extracts



- **Purification of AAV2 from cell pellets**
  - Four flasks transfected – biological replicates
- **Extraction of ssDNA from AAV2 particles**
  - AAVpro Takara Bio kit
- **Quantify with dPCR**
  - Separate the ITRs using *Msp*I (digests ssDNA)
  - ITRs have secondary structure on ssDNA
- **Calculate gene ratios**
  - In general, EGFP < bGH PolyA
  - ITR copy number is higher than EGFP and PolyA
  - Digestion with *Msp*I increases ITR copy number

*pAA2 transfer on relative scale for illustrative purposes

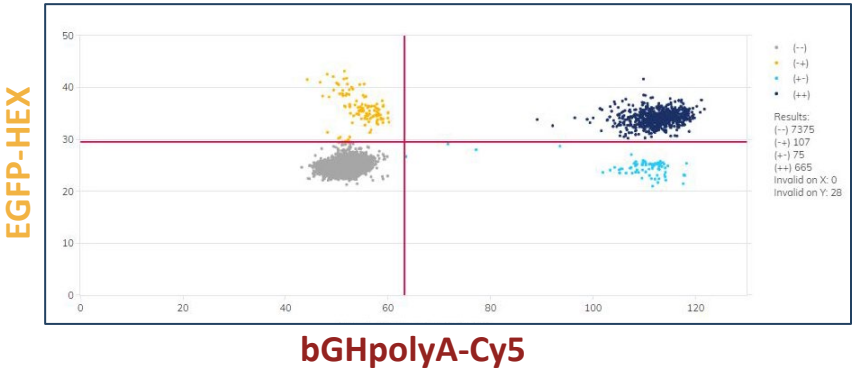
Develop multiplex dPCR method

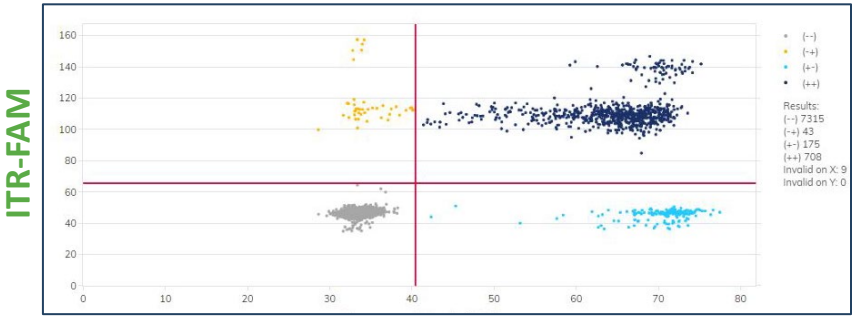
- **Multiple targets across the ssDNA**
  - Selected a five-colour instrument - QIAcuity
  - Designed two more assays

Linearised plasmid

  - ITR-FAM
  - EGFP-HEX
  - WPRE-Atto550
  - CMV-ROX
  - bGH PolyA-Cy5
  - Verified the assays worked in uniplex

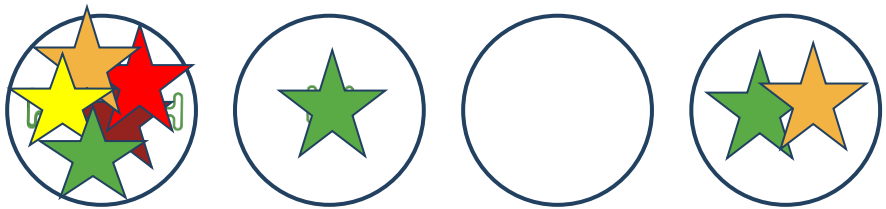
- **"Plug and play" approach**
  - SV40 PolyA-Cy5
  - Other promoters

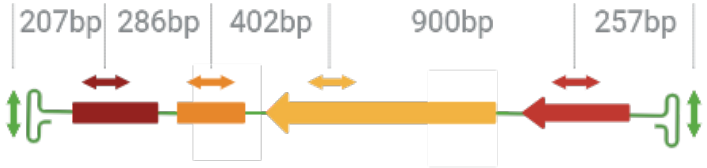


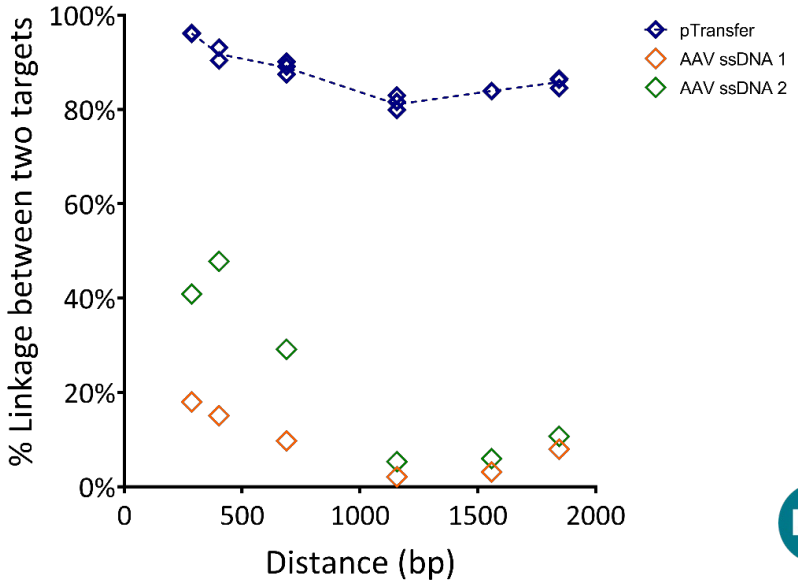


Co-segregation of targets in one partition

- **Exploit the linkage of the targets in the AAV genome**
  - Identify the proportion of targets that co-segregate
    - a partition more frequently contains two or more targets than by chance
    - Regan et al (2015) PLoS ONE 10(3): e0118270

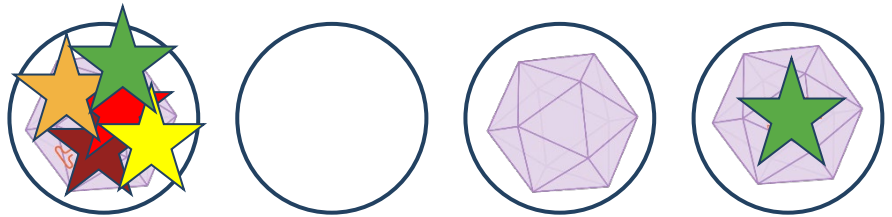


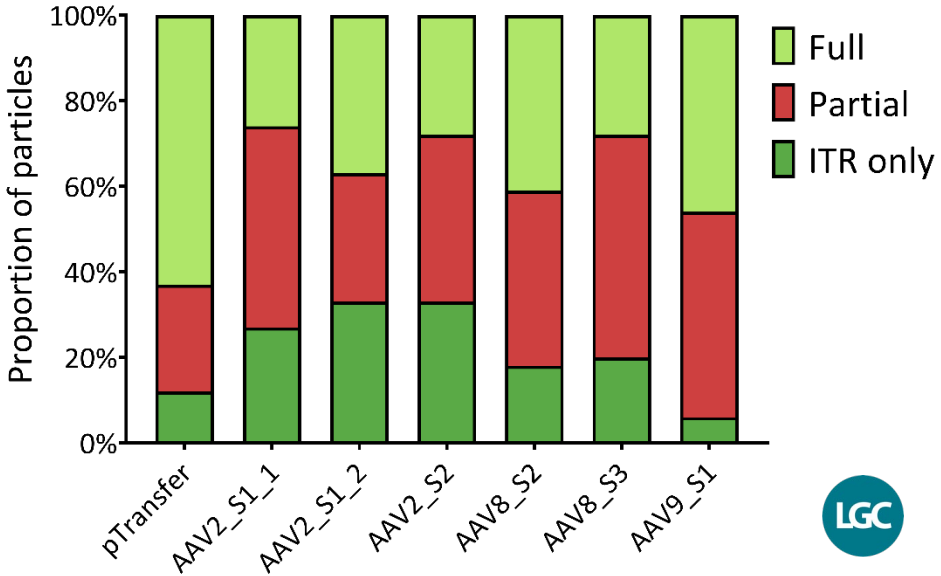




Characterisation of viral vector particles

- **Direct addition of the viral particles into the dPCR**
  - One viral particle per partition – enable presence/absence of all the targets per particle
  - Low concentration ~10% partitions contained a particle (low chance of 2+ particles per partition)
  - After partitioning, capsids burst with a 95 °C for 10 min before PCR
  - Multiplex assays; no *Msp*I added
- **Different sources of AAV (S)**
- **Different serotypes of AAV**
  - AAV8
  - AAV9





Summary

- **dPCR is a powerful tool that can be used to characterise the nucleic acids at various stages of the production of viral vectors**
- **Challenge of bespoke nature of viral vectors**
  - dPCR method can be developed using the pTransfer vectors
  - Newer platforms can enable greater multiplexing – more targets to cover the genome
    - Multiplexing is reasonably uncomplicated
    - Straightforward to validate the process and identify main sources of error
- **Method developed using the pTransfer was transferred directly to measure**
  - Extracted ssDNA
  - Whole AAV particles
  - Initial look at the “plug and play” strategy (using the polyA target initially) enabled us to look at other seratypes of AAV

Future work….

Digital PCR as a reference measurement procedure for counting viral genomes	Absolute quantification by counting molecules based on the nucleotide sequence
Support method development	Transduction efficiency, potency, transgene expression, genome integration
Purity (carry over from the production)	

Acknowledgements

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