Relative Infectivity as a Reliable Alternative to the $TCID_{50}$ Assay

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AAV gene therapies for rare diseases

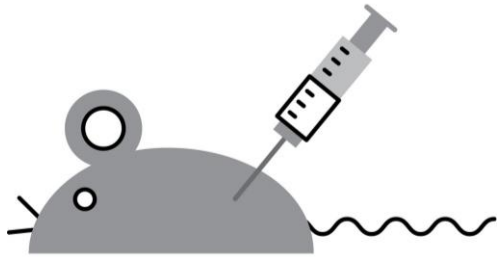
- **The recent approvals of Luxturna™ and Zolgensma® have provided validation for the use of AAV as a gene delivery platform, especially in the treatment of rare genetic diseases**

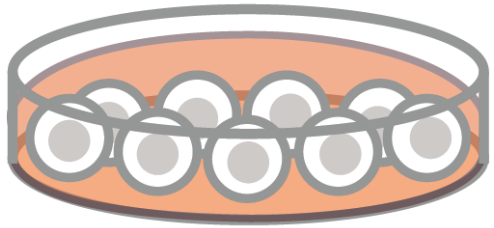
- **For rare diseases, early clinical success may lead to expedited product development and approvals**

- **Product approval requires a potency assay that:**
  - Measures the biological activity or activities specific to the product
  - Is validated to be accurate, sensitive, specific, and reproducible
  - Includes appropriate reference materials, standards, and/or controls
  - Provides quantitative data that can indicate stability, in order to establish dating periods

- **Development of a potency assay is also critical for supporting product comparability and facilitating product development**

Comparison of *in vivo* vs. *in vitro* approaches for AAV potency assay development





In Vivo

⇔ **Can be designed to represent most aspects of the product's mechanism of action, depending on the selected animal model**

⇩ **High biological variability**

- Limited quantitative ability
- Limited sensitivity for detecting changes upon stability

⇩ **Long time to results**

- Results can take months

In Vitro

⇔ **Can be designed to represent many aspects of the product's mechanism of action, including transfer and biological effect of the target transgene**

⇧ **Lower biological variability**

- Capable of providing quantitative results, relative to well-characterized reference standard
- More sensitive to changes upon stability

⇧ **Shorter time to results**

- Results may be turned around in a week

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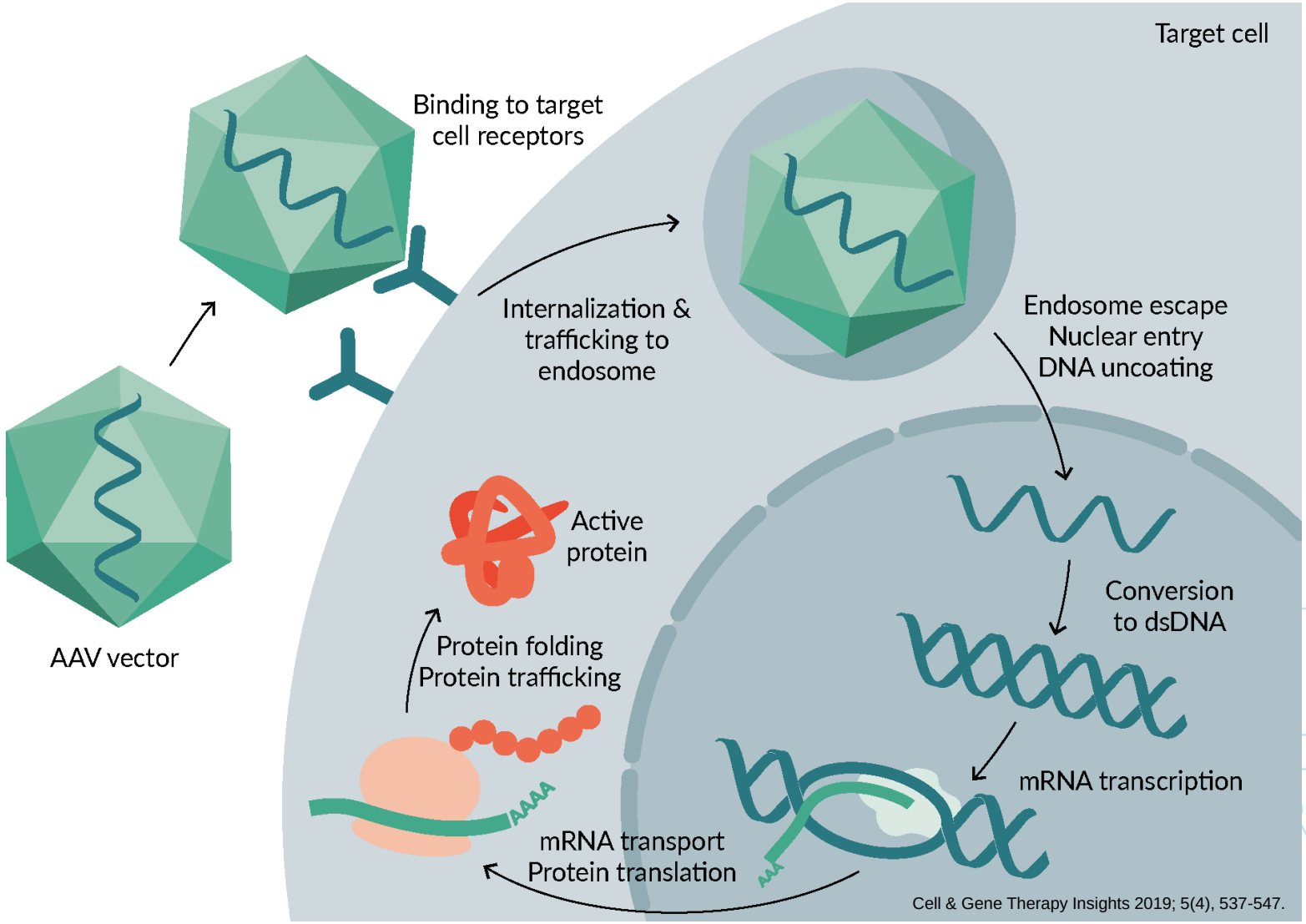
Multiple coordinated steps are required for AAV transduction

- Problem: *In vitro* methods that measure delivery of the transgene as well as the biological effect of the expressed sequence can take extensive time and effort to develop, which can slow the pace of product development
- Solution: Use a quantitative platform *in vitro* method that measures transgene delivery (infectivity) during early product development, along with less quantitative methods that confirm biological activity
  - Allow time for development of the *in vitro* potency method



Cell & Gene Therapy Insights 2019; 5(4), 537-547.

Measuring infectivity for rAAV

- **Virus infectivity: The capacity of viruses to enter the host cell and exploit its resources to replicate and produce progeny infectious viral particles**

- **Traditional virological methods were developed 60-100 years ago to quantify infectious virus particles using cells permissive to infection *in vitro***
  - Infectious center assays (i.e., plaque, focus forming assays)
  - Endpoint dilution assays (i.e., median tissue culture infectious dose or $TCID_{50}$, most probable number or MPN)

- **However:**
  - wtAAV requires a helper virus for replication
  - rAAV gene therapy products lack the *rep* and *cap* genes and are incapable of replication, even in the presence of helper virus
  - Different AAV serotypes display differences in tissue and cell tropism

- **The $TCID_{50}$ assay was modified for AAV in order to accommodate the traditional definition of virus infectivity:**
  - HeLa RC32 cells stably expressing AAV2 *rep* and *cap* genes
  - Co-infection with wild-type Adenovirus 5 helper virus
  - Instead of cytopathic effect (CPE), wells are scored as infected or uninfected based on the measurement of vector genome replication

The $TCID_{50}$ infectious titer method

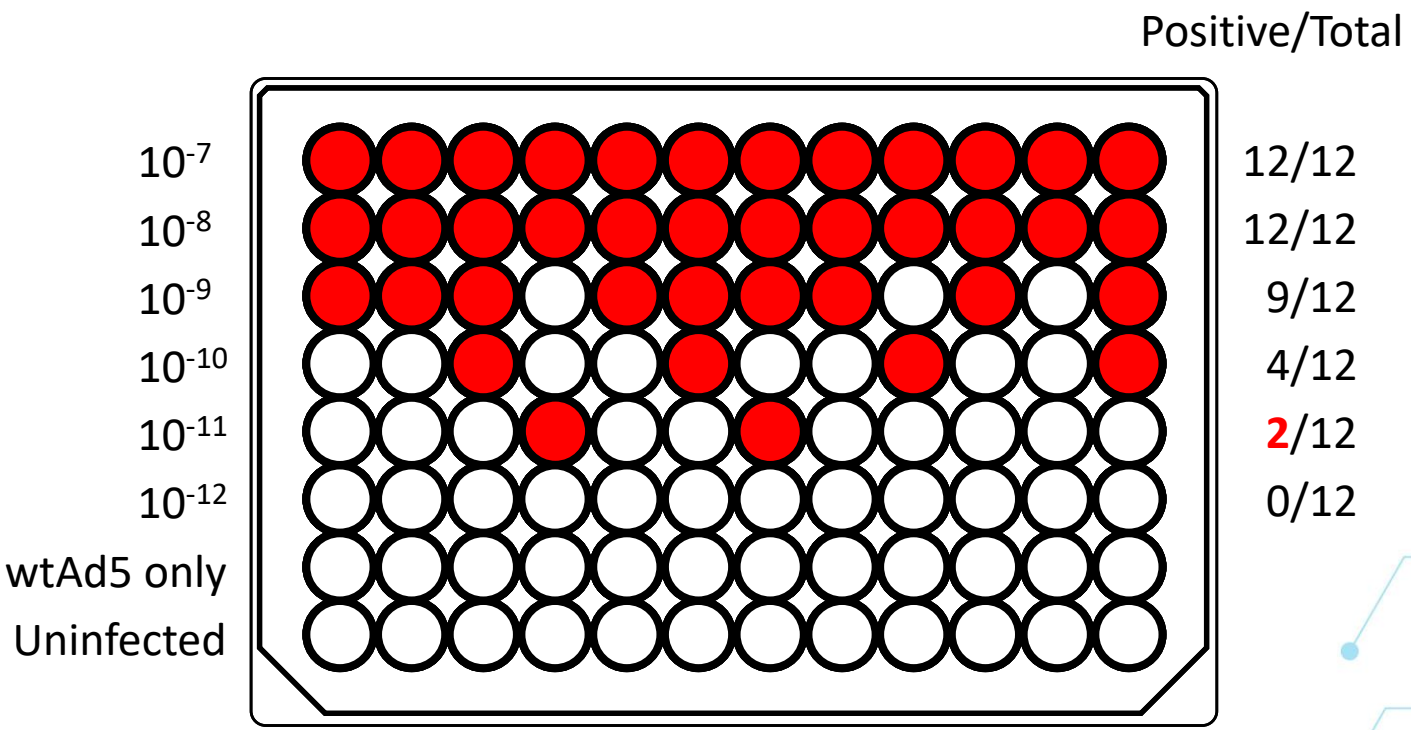
Seed HeLa RC32 cells

1 day

Inoculate cells with 10-fold serial dilutions of AAV with wtAd5
(multiple wells for each dilution)

3 days

Lyse cells, extract DNA, perform qPCR
Calculate IU/mL titer based on the number of infected wells at each dilution (Spearman-Karber Method)



10.75 log_{10}IU/mL

$5.62x10^{10}$ IU/mL

(20% Difference)

The $TCID_{50}$ infectious titer method has very high assay variability

- ~200% geometric coefficient of variation (CV) is typical for $TCID_{50}$
- $TCID_{50}$ is an unreliable tool for measuring differences in infectivity across different vector preparations or changes as a result of degradation

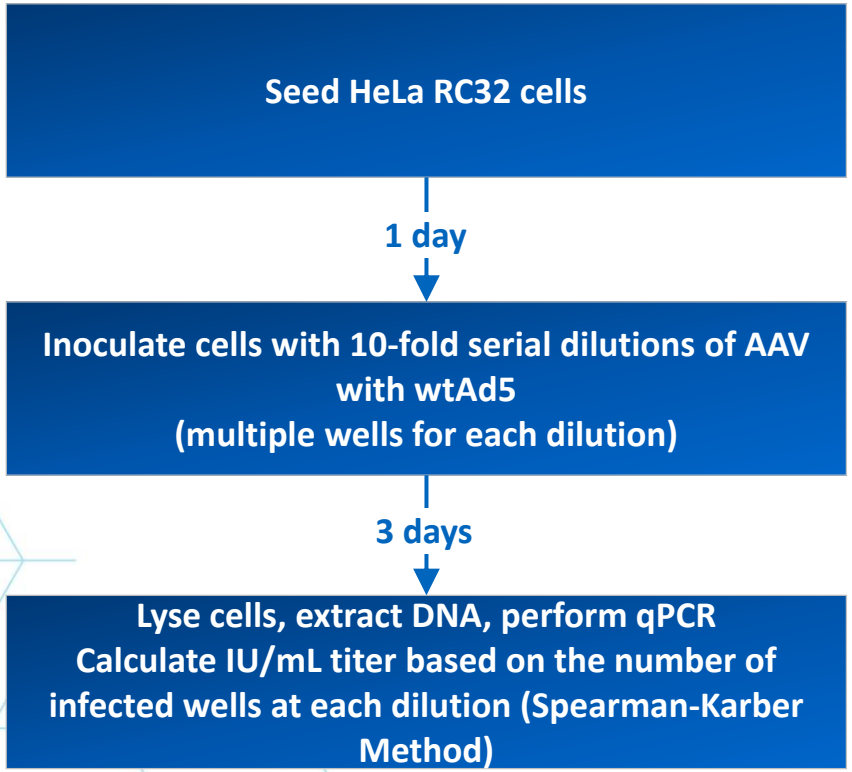


TABLE 5. FINAL rAAV2 REFERENCE STANDARD MATERIAL TITER ESTIMATES AFTER TRANSFORMATION AND MODELING

Titer units (method)	*Transformation*[a]	*Mean*	*Lower 95% confidence limit for the mean*	*Upper 95% confidence limit for the mean*	*±2 SD*	*±3 SD*
Particles/ml (ELISA)	Untransformed	9.18×10^{11}	7.89×10^{11}	1.05×10^{12}	3.73×10^{11}–1.45×10^{12}	1.04×10^{11}–1.78×10^{12}
Vector genomes/ml (qPCR)	Square root	3.28×10^{10}	2.70×10^{10}	4.75×10^{10}	9.00×10^{8}–1.04×10^{11}	0–1.66×10^{11}
Transducing units/ml (green cells)	Square root	5.09×10^{8}	2.00×10^{8}	9.60×10^{8}	0–2.47×10^{9}	0–4.00×10^{9}
Infectious units/ml ($TCID_{50}$)	Log_{10}	4.37×10^{9}	2.06×10^{9}	9.26×10^{9}	5.15×10^{8}–3.71×10^{10}	1.77×10^{8}–1.08×10^{11}

SD = 0.46 log_{10} IU/mL → 191% Geometric CV

TABLE 4. FINAL rAAV8 REFERENCE STANDARD MATERIAL TITER ESTIMATES AFTER TRANSFORMATION AND MODELING

Titer units	*Transformation*	*Mean*	*Lower 95% confidence limit for the mean*	*Upper 95% confidence limit for the mean*	*±2 SD*
Particles (pt)/ml	None	5.5×10^{11}	4.26×10^{11}	6.75×10^{11}	1.06×10^{11} to 9.94×10^{11}
Vector genomes (vg)/ml	Log_{10}	5.75×10^{11}	3.05×10^{11}	1.09×10^{12}	4.57×10^{10} to 7.24×10^{12}
Infectious units (IU)/ml	Log_{10}	1.26×10^{9}	6.46×10^{8}	2.51×10^{9}	1.32×10^{8} to 1.20×10^{10}

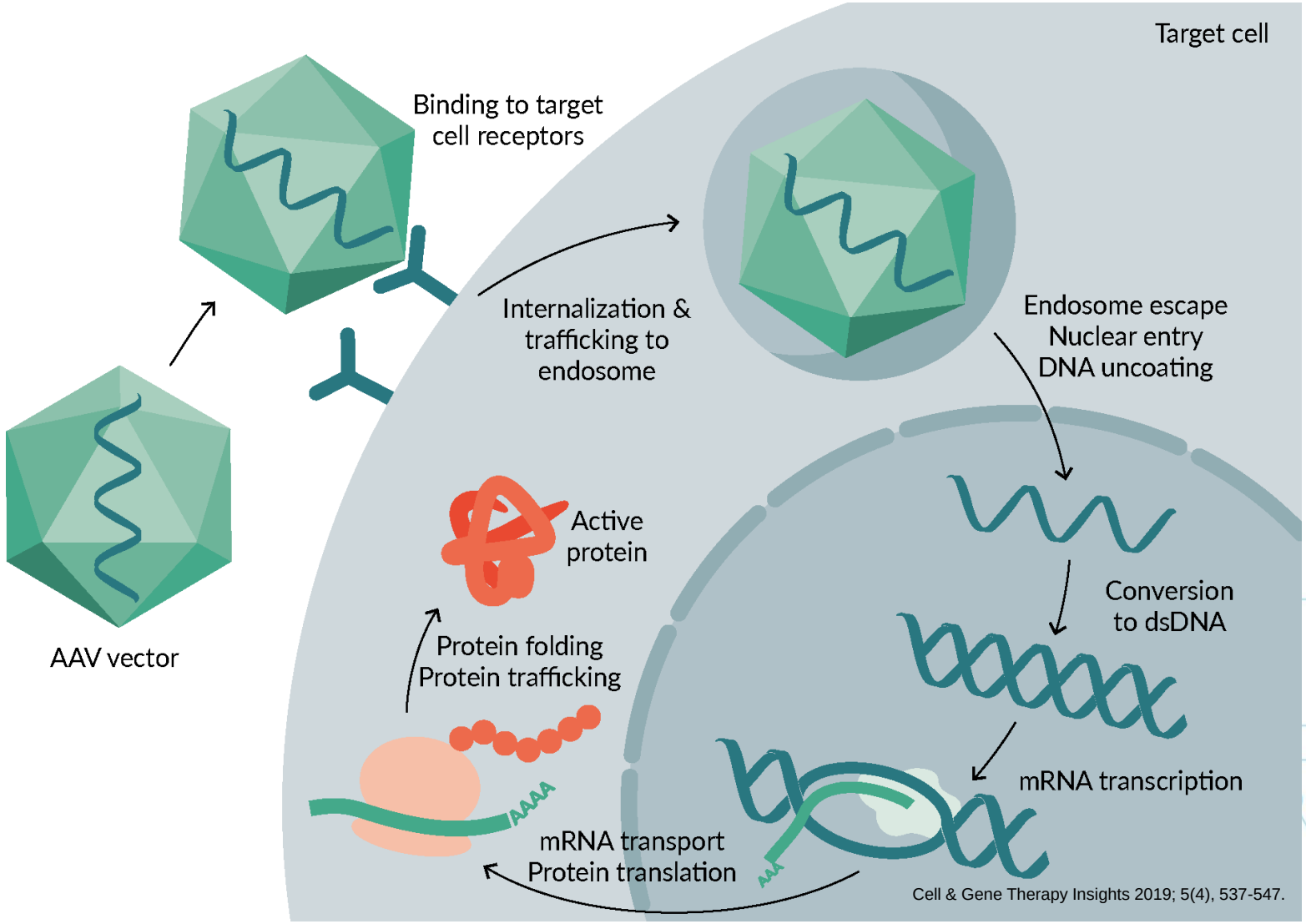
SD = 0.49 log_{10} IU/mL → 209% Geometric CV

Sources: Human Gene Therapy 2010; 21(10), 1273-1285. Human Gene Therapy 2014; 25(11), 977-987.

How can you measure rAAV infectivity with better precision?

- **Since rAAV cannot replicate, rAAV infectivity should be defined as the capacity of rAAV to enter the target cell and deliver its genome**

- **Measure delivery of the AAV vector genome to target cells relative to a reference standard**



The relative infectivity method as a reliable alternative to the $TCID_{50}$ method

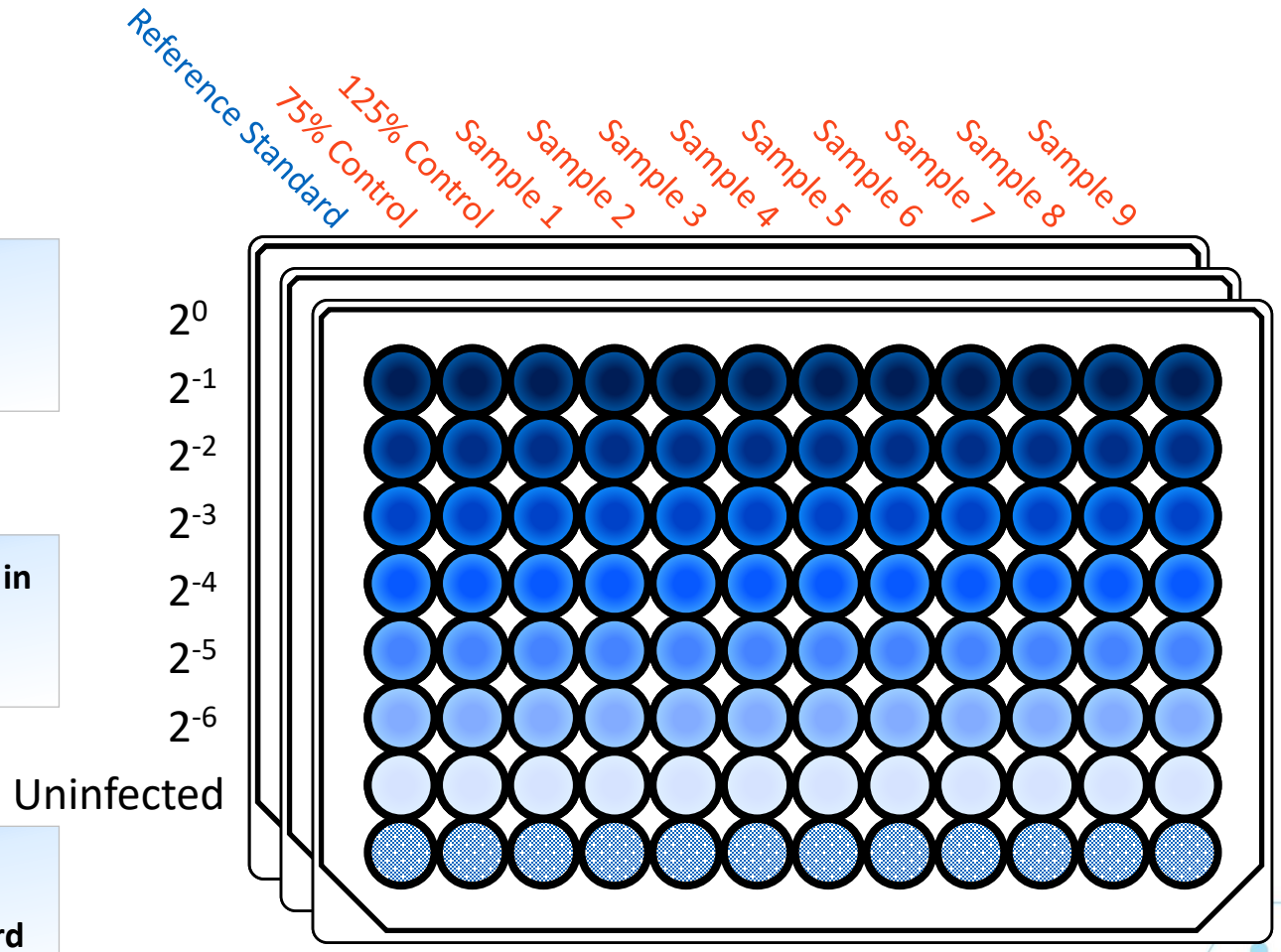
Seed desired target cells

1 day

Inoculate cells with 2-fold serial dilutions of AAV in parallel with well-characterized AAV reference standard

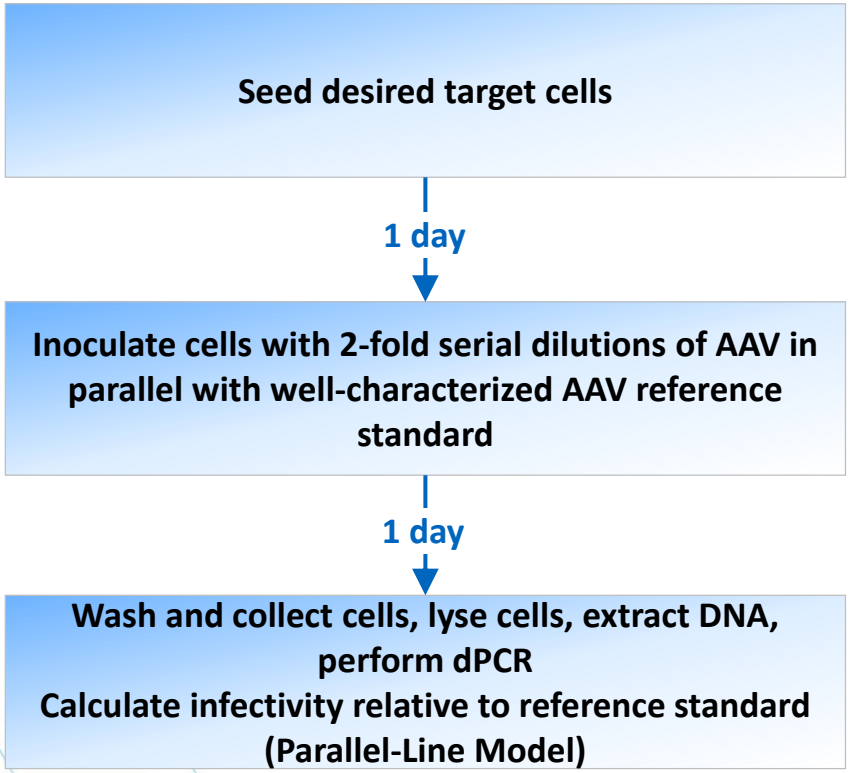
1 day

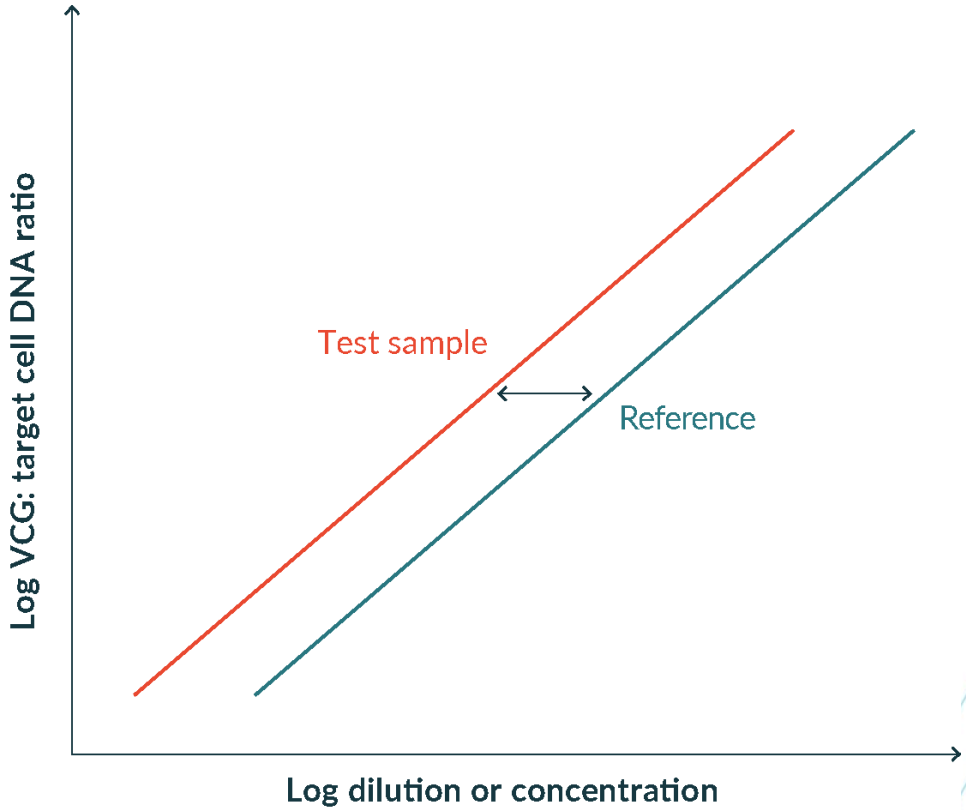
Wash and collect cells, lyse cells, extract DNA, perform dPCR
Calculate infectivity relative to reference standard (Parallel-Line Model)



The relative infectivity method as a reliable alternative to the $TCID_{50}$ method

- Faster time to result than $TCID_{50}$ (2 days vs 4 days)
- Requires a well-characterized AAV reference standard that is known to be infectious
- Does not require co-infection with wild-type Adenovirus





VGC: Vector genome concentration

Source: Cell & Gene Therapy Insights 2019; 5(4), 537-547.

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Calculate VGC:target cell DNA ratio for each dilution of reference and test sample

↓

Plot VGC:target cell DNA ratio vs. log dilution for the reference and test sample

↓

Check that plots for reference and test sample are linear and similar (parallel)

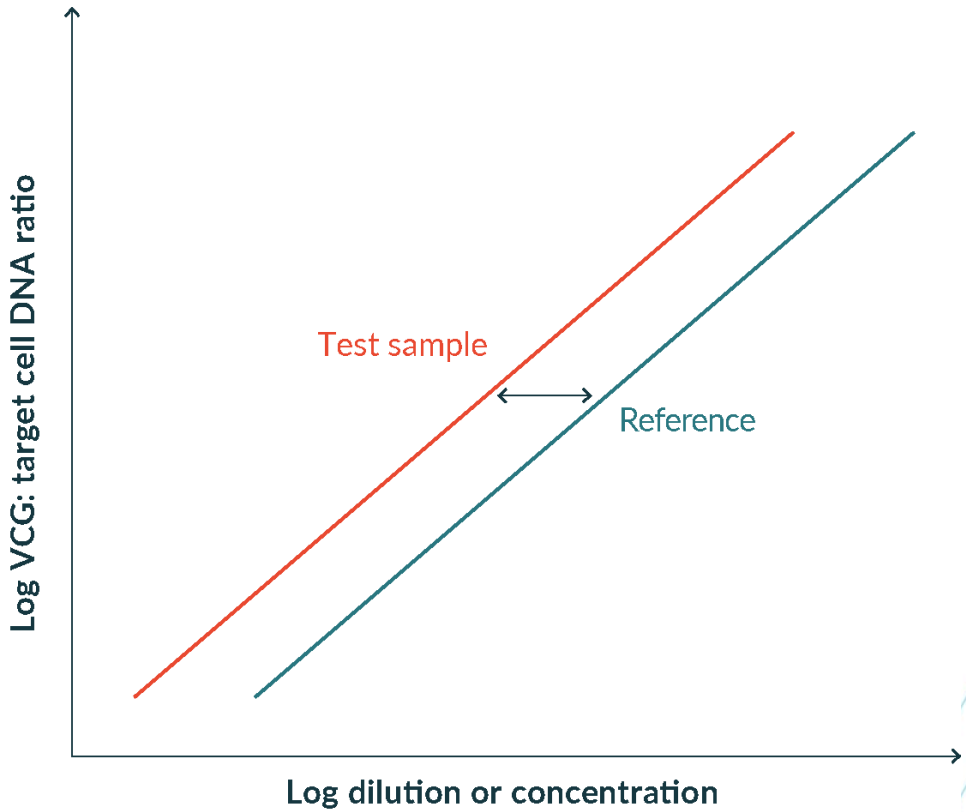
↓

Perform constrained fit of reference and test sample (common slope)

↓

Calculate relative infectivity (%)

$$antilog\left(\frac{Intercept_{Test\ Sample} - Intercept_{Reference}}{Common\ Slope}\right)$$



VGC: Vector genome concentration

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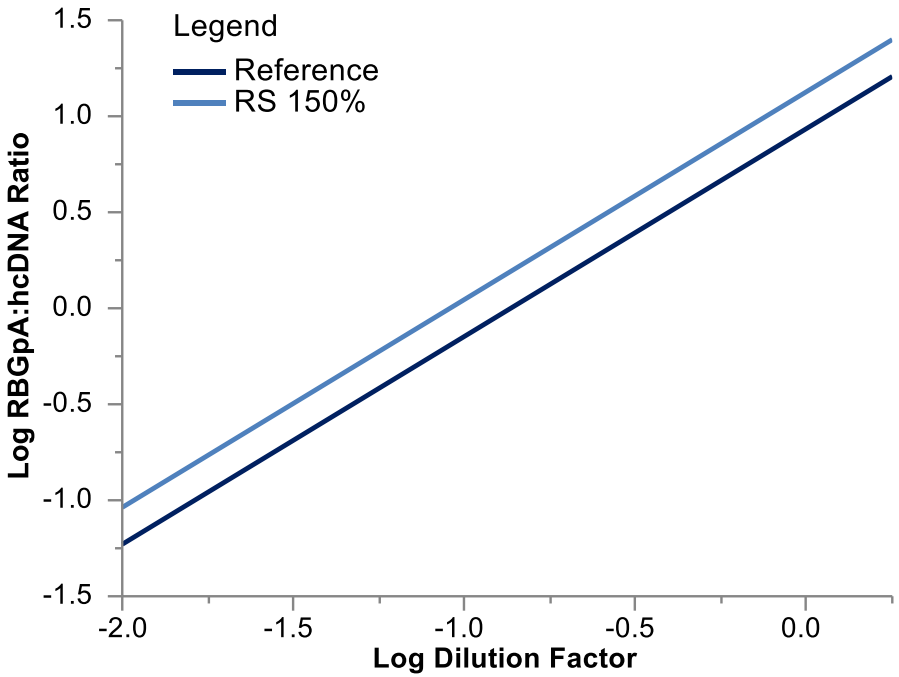
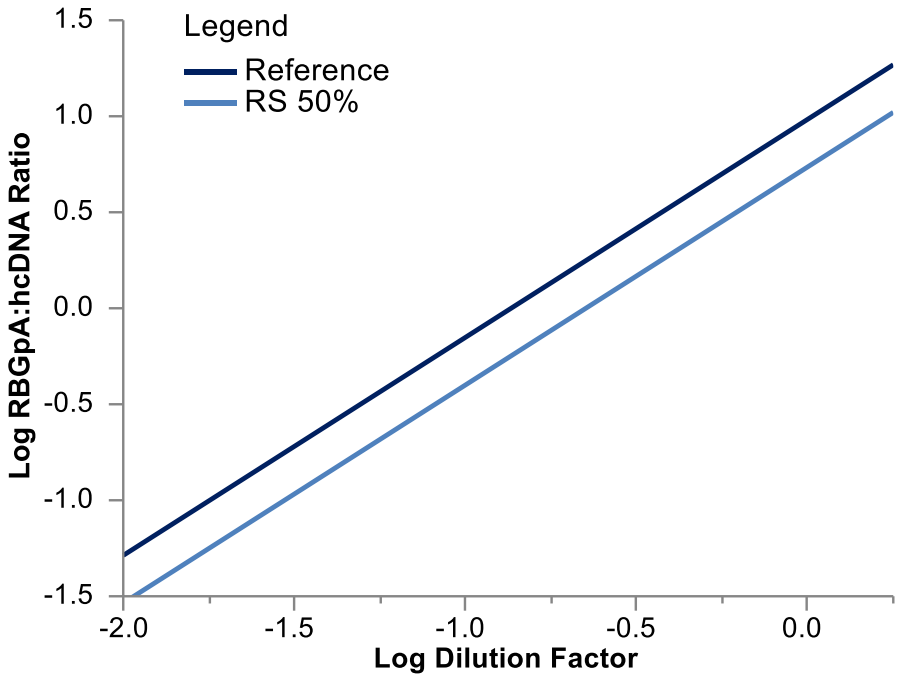
Example relative infectivity method results using reference material



Expected Value = 150%

Measured Result = 150.4%



Expected Value = 50%

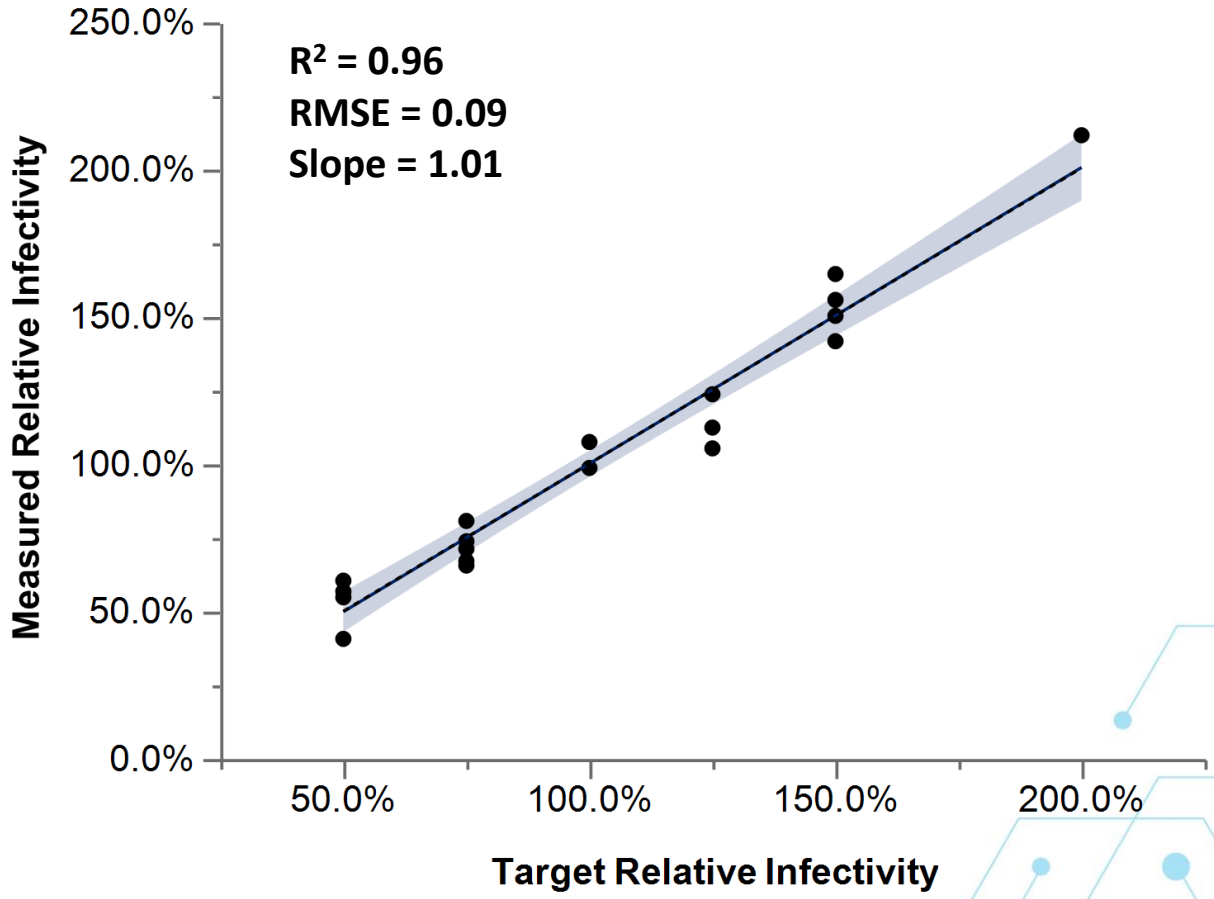
Measured Result = 56.9%

The relative infectivity method is linear, accurate, and precise

- **The relative infectivity method is capable of quantifying relatively small differences in the *in vitro* infectivity of AAV vectors**

Target Relative Infectivity	Measured Relative Infectivity	% Accuracy
200%	212%	106%
150%	153%	102%
125%	114%	91%
100%	103%	103%
75%	72%	96%
50%	55%	109%
	Overall %CV	**11%**

Tested using rAAV8 material (N = 20, collected over nine different runs)



Applications of the relative infectivity method

- **Comparisons across different products**

	Serotype	Relative Infectivity
Product A	AAV9	124%
Product B	AAV9	75%
Product C	AAV9	78%
Product D	AAV8	89%

Note: Different dPCR methods were used for the different products

- **Comparisons across multiple batches of the same product (i.e., product comparability)**

	Batch / Lot	Relative Infectivity
Product D	1	103%
Product D	2	108%
Product D	3	81%
Product D	4	102%
Product D	5	90%

Applications of the relative infectivity method

- **Detect changes upon stress & stability**

Condition		Relative Infectivity
Untreated Control	-80°C	99%
Thermal Stressed	60°C for 10 minutes	375%

- **Compare the ability of vectors to infect different cells & conditions**

Target Cells	Relative Infectivity
HEK293 (Reference)	100%
HEK293 with Modification A	147%
HEK293 with Modifications A and B	6,968%

- **Assess improvements in infectivity for engineered AAV capsid variants**
- **Probe AAV infection kinetics**

Limitations of the relative infectivity method

- **Accurate quantitation of the vector genome concentration is required for the test samples and the reference standard**
- **The use of a well-characterized reference standard with known biological activity or infectivity is critical**
- **The method is intended to measure intracellular vector genomes, and therefore does not provide a measure of target protein expression or biological activity of the transgene**

Conclusions

- We have developed a platform-based *in vitro* relative infectivity method that is capable of detecting differences as low as 25% in the infectivity of AAV vectors, representing a significant improvement over $TCID_{50}$
- The relative infectivity method is linear, accurate, and precise from at least 50-200% relative infectivity
- The relative infectivity method is capable of detecting a change in infectivity upon forced degradation
- The relative infectivity method may be a useful tool in early development for comparing infectivity across different preparations, products, serotypes, and target cells
- In the absence of a quantitative product-specific *in vitro* potency method, the relative infectivity assay is a more reliable tool than $TCID_{50}$ for supporting product comparability and monitoring product stability
- The relative infectivity method may provide a measure of product potency for early phase development until a quantitative product-specific in vitro potency method can be developed and implemented

Acknowledgments

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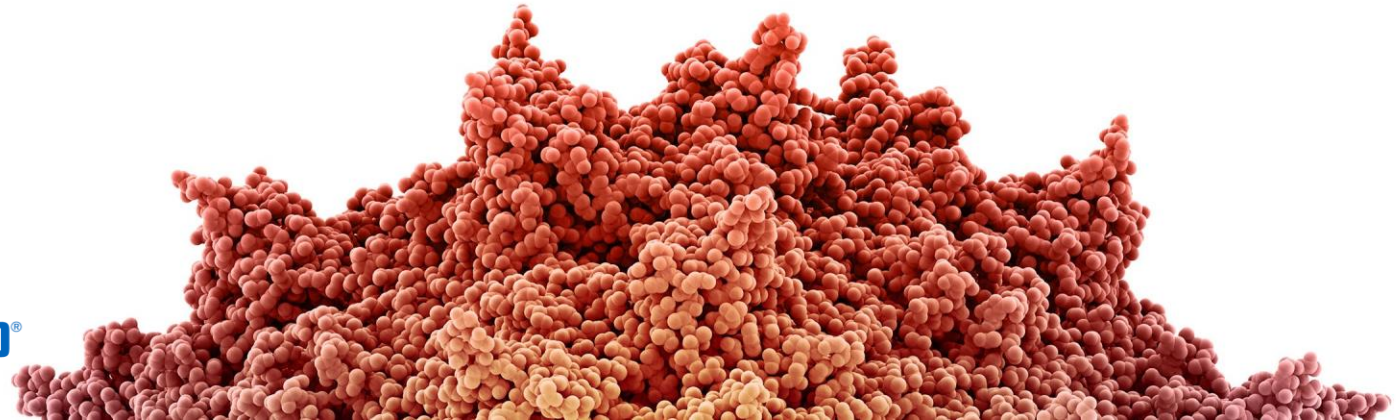

Thank You

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