PCR-based Detection of rAAV Vectors in WBCs Transduced Collaterally *in vivo*: Application to Gene Doping Surveillance

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Recombinant Adeno-Associated Viral (rAAV) Vectors

- Based on a human parvovirus which causes no known disease
- 12 Serotypes and over 100 genetic variants, hybrid capsids
  - Preference for different organ targets
- Normal, mature, and quiescent tissue transduced efficiently
  - variety of tissue and cell types
- No obvious toxicity or pathology following transduction
- Ability to be stably maintained
  - A single administration of AAV vector results in efficient gene transfer in a dose dependent manner
  - Transgene expression persists for years
  - Vector Genome persists as a circular episome
- Human trials for therapy
  - Efficacy seen for inherited blindness, hemophilia, and AADC Deficiency

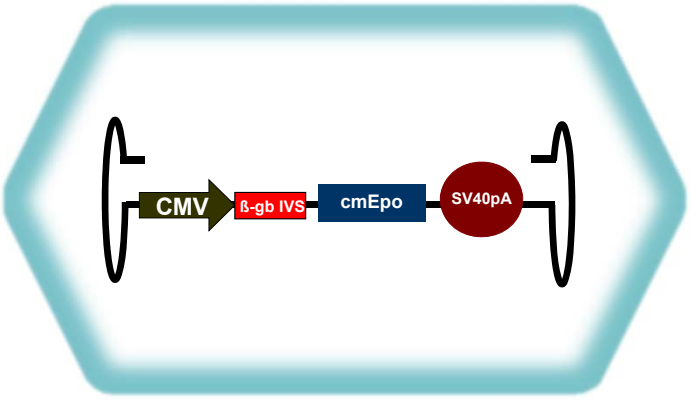
Goals

Develop sensitive and specific PCR assays to detect vector genomes potentially used in illegitimate gene transfer

- Samples collected minimally-invasively: blood, serum, or urine
- Minimal sample processing (ie avoid PBMC purification on Ficoll)
- Vector DNA extracted and captured efficiently and reliably
- Real-time PCR
- Simple testing format and analysis for surveillance
- Central data collection and analysis

rAAV-cmEPO vector

Strong promoter for low vector dose



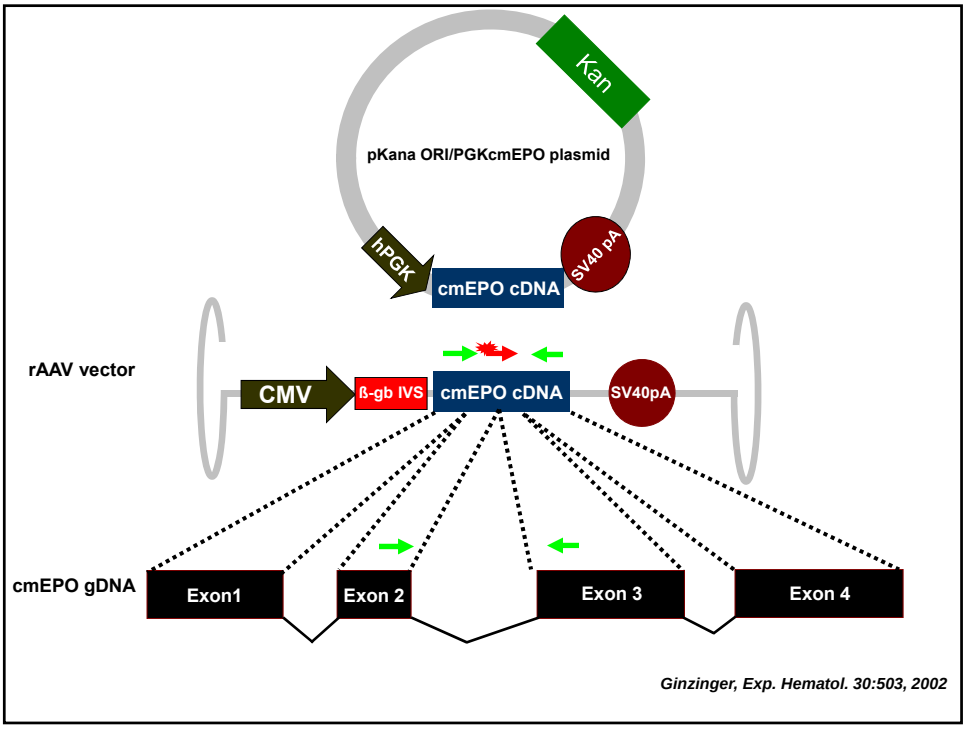
Real-time Taqman PCR Assay Development

Six Assays

1. Kanamycin
2. SV40 pA
3. **cmEpo Exon2-3**
4. ε-Globin
5. **hEPO Exon3-4**
6. CMV promoter

Optimizing PCR conditions

- Primers-Probe set design
- Primer+probe concentration
- Master Mix
- Program parameters



Ginzinger, Exp. Hematol. 30:503, 2002

Real-time Taqman PCR Assays

- Linearity is maintained over 8-logs
- Genomic DNA (gDNA) does not significantly interfere or compete
- Sensitivity of cmEPO Exon assay is 3 copies
- No false-positives from naïve gDNA
- Sensitivity of Kan and SV40 assays is 3 copies
- Single-step PCR (not nested)

Other Aspects

- Recovery of episomal vector molecules in the presence of gDNA
- Integrity of DNA: Isolation, Storage, and Shipping
  - OD 260/280 and OD 260/230
  - Gel electrophoresis
  - ε-globin PCR
- Remote data collection into Central Repository
  - Real-Time PCR machines are internet ready

Rationale

Compared to rodents, NHP more closely compare to human beings in muscle structure blood volume to mass ratio, viral receptors and immune response

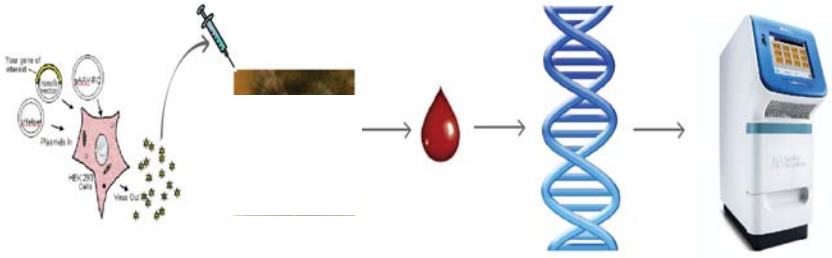
Determine the efficiency of vector escape from the injection site into the circulation

Low dose to slightly raise the hematocrit and determine the sensitivity of detecting vector sequences in relation to dose

Evaluate rAAV vectors after IM administration

- Biodistribution vs Dose
- Biodistribution vs rAAV Serotypes
- Biodistribution vs Time
- Relationship between dose and WBC signal

Experimental design for *in vivo* Samples

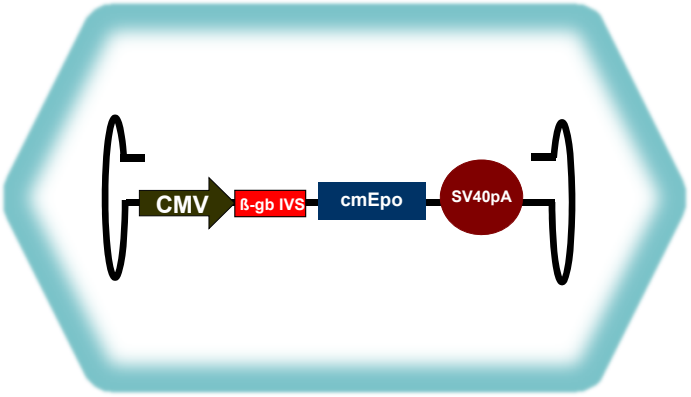


Dose for Hct slightly above normal

Analyze 500ng = 75,000 WBC (=15 uL of blood)

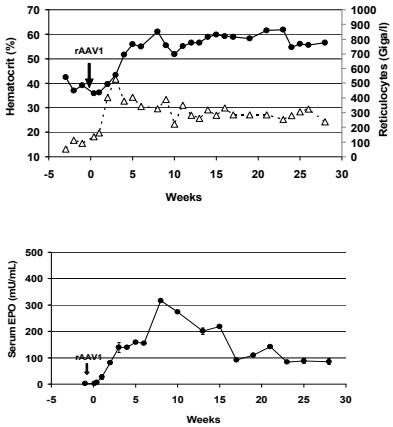
Vector Administration

- Mac 3: rAAV1 at 2.5E10 vg/kg (= 7.75E10vg)
- Mac 4: rAAV1 at 2.5E11 vg/kg (= 9E11vg)
- Mac 5: rAAV8 at 5E9 vg/kg (= 1.3E10vg)
- Mac 6: rAAV8 at 2.5E10 vg/kg (= 1.01E11vg)



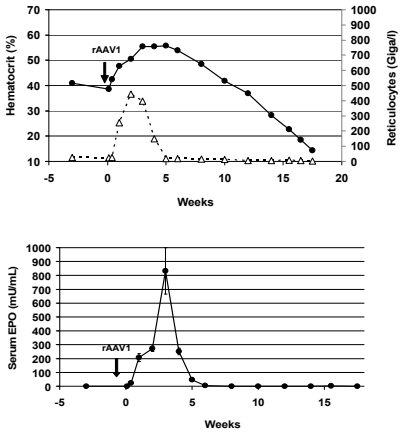
rAAV1 Administration

Mac 3



2.5E10 vg/kg (= 7.75E10vg)

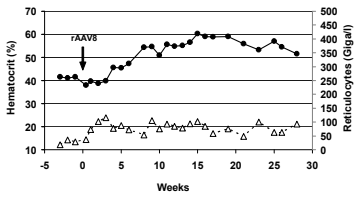
Mac 4

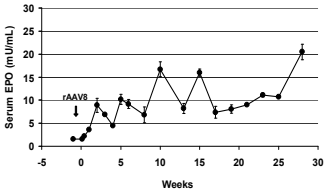


2.5E11 vg/kg (= 9E11vg)

rAAV8 Administration

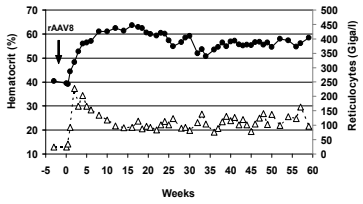
Mac 5

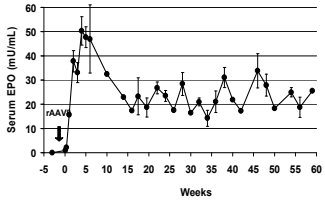




5E9 vg/kg (= 1.3E10vg)

Mac 6

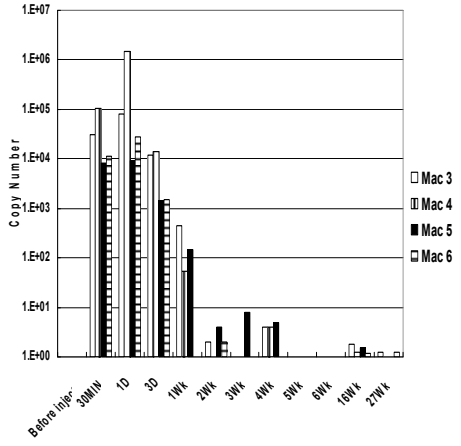




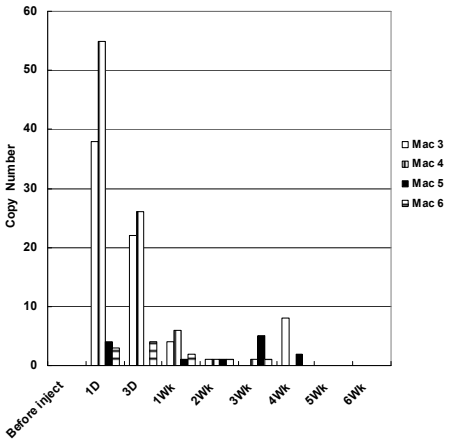
2.5E10 vg/kg (= 1.01E11vg)

PCR Detection of rAAV in NHP Serum and Urine

Serum

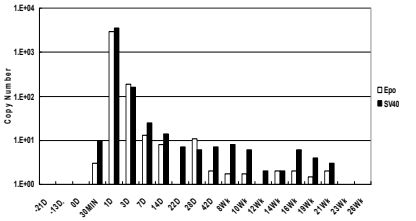


Urine

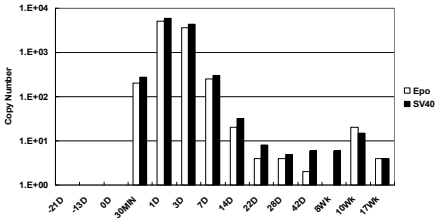


Presence of rAAV Genomes in NHP WBCs

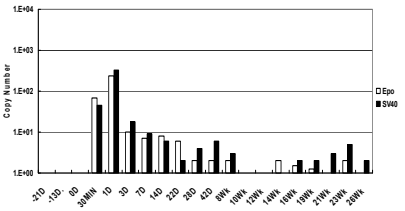
Mac 3 - rAAV1



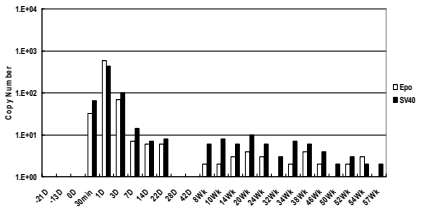
Mac 4 – rAAV1



Mac 5 – rAAV8



Mac 6 – rAAV8

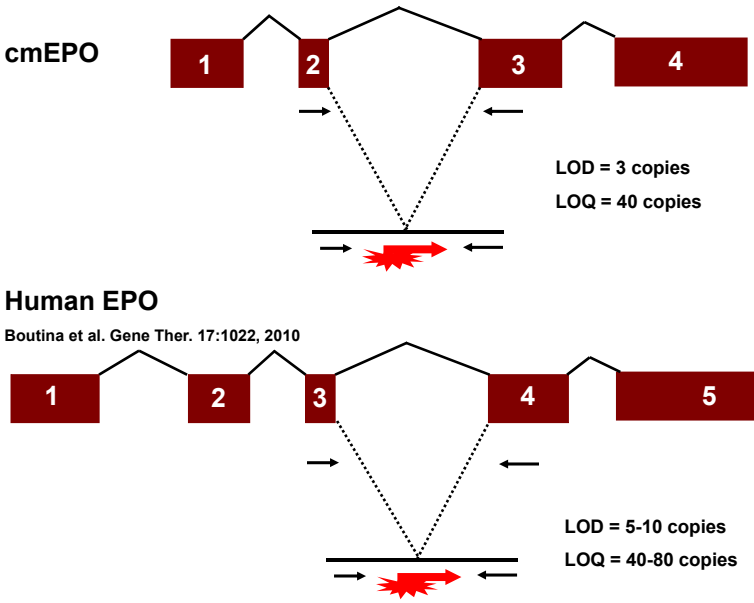


Conclusions

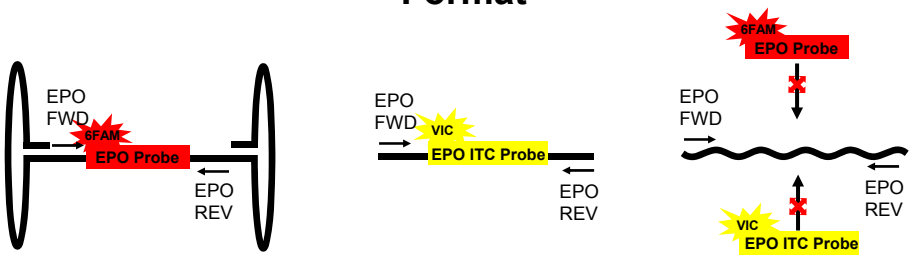
rAAV Administration

- PCR Assays perform in testing of different tissue samples from animals transduced *in vivo*
- rAAV detected in serum and urine up to one month pi
- WBCs are collaterally transduced by rAAV following IM administration
- rAAV vector genome is maintained long-term in a small population of WBCs
- >95% of rAAV vector genomes in WBC are circles at d3 post-administration and persist long-term (unpublished data)

Human Assay Design



Internal Threshold Control (ITC) Duplex Assay Format



rAAV vector | **synthetic ITC^{EPO} template** | **genomic EPO locus**

Unknown Copy Number	Known Copy Number	
C_T >	C_T	Negative
C_T ≤	C_T	Positive

Internal Threshold Control (ITC) Testing Format

gDNA Sample

A defined number of ITC template molecules

PCR Reagents

- Single tube: gDNA + master mix (primers, probes and ITC template)
- Specific and sensitive, all samples INTERNALLY controlled (no false negatives)
- No standard curve titration tubes (NO EXTERNAL STDS)
- High throughput: many samples analyzed at once
- Reduced risk of contamination (false positives)
  - No positive control plasmid needed
  - PCR product
    - uracil-N-glycosylase (UNG) prevents the reamplification of carryover PCR products in subsequent analyses

ITC^{hEPO} Duplex Assay Testing

Copy Number of Spiked Plasmid	Mean hEPO Ct (s.e.)	Mean ITC^{hEPO} Ct (s.e.)	Positive/Negative
0	40.00 (0)	36.93 (0.42)	Negative (P-value <0.0001)
188	32.60 (0.44)	36.48 (0.33)	Positive (P-value <0.0001)
13	36.63 (0.28)	37.12 (0.27)	Positive (P-value =0.2426)
8	36.88 (0.28)	36.84 (0.28)	Positive (P-value =0.9172)
2	39.80 (0.20)	37.32 (0.37)	Negative (P-value <0.0001)

n=5, 10 copies of ITC^{hEPO} template

Analytical Validation Parameters
(Real-time PCR Approach)

• Precision	• CVs of less than 3%
• Specificity	• No false positives
• Limit of Detection	• LOD: 5-10 copies
• Limit of Quantitation	• LOQ: 40-80 copies
• Linearity	• R^2 approach 1
• Range	• 8 logs
• Ruggedness	• Different labs, instruments
• Robustness	• Deliberate variations

Progress

- Real-time PCR assays with high specificity and sensitivity have been developed
- Capable of detecting a low number of vector copies in NHP WBCs many months following administration IM
- An ITC assay format that is automated, reliable, and has a binary (positive/negative) readout facilitates high throughput and rapid sample surveillance at low cost
- Technology can detect different types of gene transfer vectors

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Ni, W., LeGuiner, C., Gernoux, G., Penaud-Budloo, M., Moullier, P., and Snyder, R.O. Longevity of rAAV Vector and Plasmid DNA in Blood after Intramuscular Injection in NHP: Implications for Gene Doping. Gene Ther. 18:709-718, 2011

Ni, W., LeGuiner, C., Moullier, P., and Snyder, R.O. Development and Utility of an Internal Threshold Control (ITC) Real-Time PCR Assay for Exogenous DNA Detection. PLoS ONE. 7(5): e36461, 2012.