Development and Validation of a Duplex qPCR Assay to Study Biodistribution/Shedding of a Dual Gene Therapy Vector

Challenges and scientific considerations

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Background – Context of Use

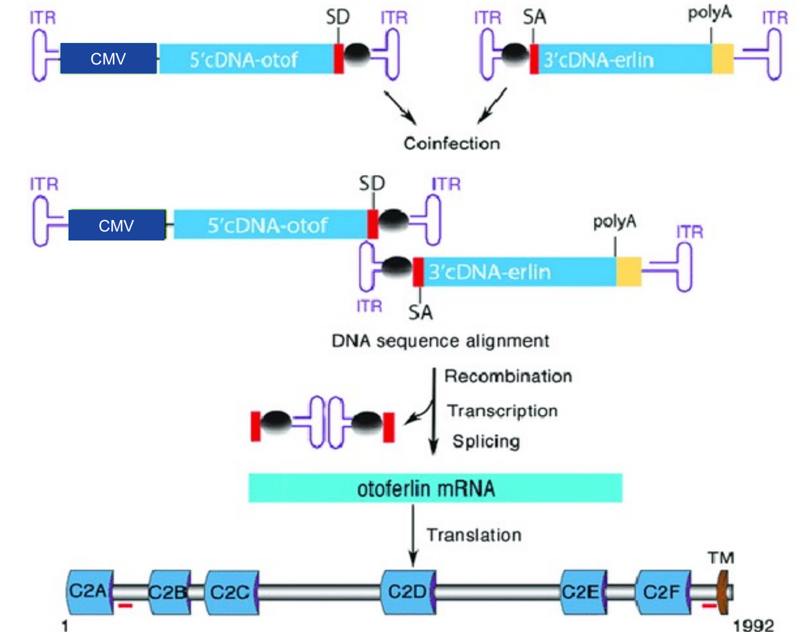
The Inner Ear Diseases company

GLP Regulatory Biodistribution and Shedding Study in Non Human Primate

Sponsor: Sensorion

Test item: OTOF-GT (SENS-501)

- Dual Gene therapy vector coding for human Otoferlin for the treatment of hearing disorders linked to Otoferlin deficiency
- Vector: AAV8
- 1:1 Mixture of SENS-501Nter and SENS-501Cter
    - Otoferlin CDS: 6 kb, too large for an AAV vector: coding sequence in 2 AAVs
    - Recombination in transduced cells leading to full-length Otoferlin coding sequence after splicing



Akil et al. Proc Natl Acad Sci U S A. 2019 Mar 5;116(10):4496-4501

Biodistribution/shedding study design: considerations for fit-for-purpose assay validation

- Single intracochlear administration, 3 and 6 months recovery periods
- Shedding: Days 2 – 4 – 6 – 9 – 16: Urine, feces, tears, saliva, nasal & ear swabs
- Tissue list for biodistribution: Vascularized tissues (ICHS12), blood, CSF and specific tissues linked to administration route: parotid and mandibular lymph nodes, auditory and optic nerves, temporal bone incl. inner ear/vestibular system

Multiple matrices with different DNA contents

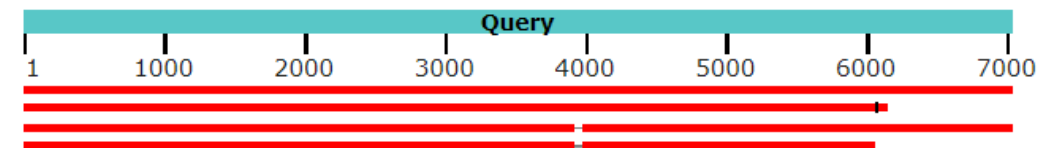
Additional specific investigations for a GT: RT-qPCR for transgene mRNA quantitation in positive tissues for DNA, nAbs, tAbs against the vector and transgene, IFN-γ ELISPOT against the vector (VP1) and transgene

Assay development strategy & challenges

Simplex first !

Primers and probe sets design for simplex assays

- Standard criteria
  - Amplicon length : 70-150 bp
  - Primers
    - Tm: 60 ± 2°C, with ΔTm < 2°C
    - GC% between 35-65%, with no GC rich region (optimal 50%)
    - Primer length: 18-30 nt
  - Probe
    - Tm > 6-8°C than primers Tm
    - Probe length: 20-30 nt
    - GC% between 35-65%



Limited number of primers/probe sets satisfying to criteria

- 97+% sequence identity between human and cynomolgus coding sequence
- Primers designed at junctions between non-coding and coding sequences for increased specificity
- 1 set per analyte with acceptable features

Experimental Design: Assay development

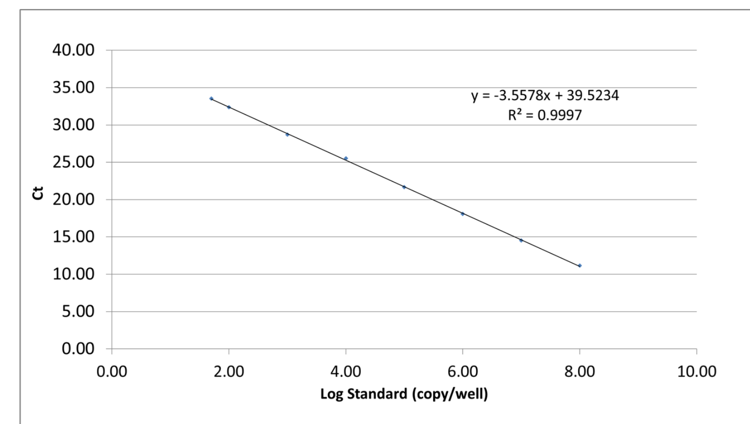
First assay performance evaluated using simplex assays, then optimization in duplex

Critical reagents

- Reference standards: linearized plasmids containing the amplicon sequence
- Primers and probe
- qPCR master mix
- Matrix

Assay design: Simplex assay optimization, then Duplex assay optimization

- Matrix selection
  - Considering multiple sample types with multiple DNA contents
  - Selection of a surrogate matrix: Herring Sperm DNA
- qPCR run assessments:
  - 8 calibration standards from 50 to 10^8 copies/well (1 µg of DNA per well when possible) in duplicates
  - At least 2 sets of QCs at 5 levels
  - Specificity in NHP genomic DNA, first evaluation of matrix equivalence between surrogate and NHP DNA
  - First estimation of P&A and PCR performance (assay linearity – PCR efficiency)
  - After assessment of simplex assays, duplex PCR assay optimization



Exemple of a calibration curve
- OTOF-Nter in duplex assay
- PCR efficiency: 91.1%
- R^2= 0.9997

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Assay validation

Adapted to the context of use and the biodistribution study specificity

No specific guideline for qPCR assay validation: validation parameters selection and acceptance criteria based on:

- Recommendations from qPCR working group of EBF : white paper followed when applicable
- GCC considerations
- Scientific considerations – adapted to the assay and the context of use : Not a bioA assay !
- Regulatory experience

Quantitative polymerase chain reaction in the bioanalytical laboratory and technical and scientific considerations for nonclinical and clinical assay characterization, validation and sample analysis

Anna Laurén[1], Manuela Braun[2], Chiara Cazzin[3], Kelly Colleti[4], Chris Cox[5], Lisa Dietz[6], Thomas Emrich[7], Kristin Geddes[8], Kate Herr[9], Tracy Iles[10], Alexandra Rogue[11], Yvan Verlinden[12] & Philip Timmerman*,[13]

Bioanalysis (2022) 14(16), 1085–1093

Recommendations on qPCR/ddPCR assay validation by GCC

Mark Wissel[1], Martin Poirier[2], Christina Satterwhite[3], John Lin[4], Rafiq Islam[5], Jennifer Zimmer[6], Ardeshir Khadang[7], Jennifer Zemo[8], Todd Lester[8], Marianne Fjording[8], Amanda Hays[8], Nicola Hughes[9], Fabio Garofolo[10], Rudolf Guilbaud[11], Elizabeth Groeber[12], Heidi Renfrew[13], Kelly Colletti[3], Mathilde Yu[14], Jenny Lin[15], Xinping Fang[16], Santosh Shah[4], Wei Garofolo*,[17], Sumit Kar[17], Roger Hayes[18], John Pirro[19], Cheikh Kane[19], Marsha Luna[19], Allan Xu[20], Stephanie Cape[21], Mark O'Dell[22], Robert Wheller[23], Hanna Ritzen[24], Jennifer Vance[25], Esme Farley[26], Katie Matys[27], Edward Tabler[27], William Mylott[27], Moucun Yuan[27], Shane Karnik[28], Troy Voelker[29], Ira DuBey[5], Clark Williard[30], Jing Shi[31] & Jim Yamashita[31]

Bioanalysis (2022) 14(12), 853–863

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Assay validation parameters & Assay performance

Analytical validation

Parameter	How ?	Result
Matrix effect	CC in surrogate matrix, sets of QC levels in surrogate and other matrices (6 sets at 5 levels), with 1 µg of DNA per well when possible	OTOF-Nter: - CV% < 20%, RE% < 45% (LLOQ) OTOF-Cter: - CV%< 18%, RE%< 22%
Precision and accuracy	CC in surrogate matrix, sets of QC at 5 levels: 6 sets (within), 6 runs with 3 sets, 2 analysts and 2 instruments (between)	OTOF-Nter: - CV% < 30%, RE% < 23% OTOF-Cter: - CV%< 25%, RE%< 22%
Quantitation limits	NA	50 to 10^8 copies/well
LOD	3 to 6 dilutions starting from LLOQ assessed in 8 replicates per run, 3 runs	OTOF-Nter: 12.5 copies/well OTOF-Cter: 3.12 copies/well (23 measurable Ct out of 24)
Dilution integrity in matrices	3 DNA dilutions tested, 3 QC levels	Passed at 60 ng/well
Assay specificity and matrix effect in blood, temporal bone, nasal swab, liver and feces DNA	5 individuals of each matrix (specificity) 5 individuals spiked at low QC	Below LOD OTOF-Cter: CV% < 27% OTOF-Nter: CV% < 34%

Assay validation parameters & Assay performance

Continued

Impact of the analyte ratio on the precision and accuracy of the assay

- Rationale: test article is a 1:1 mixture of both OTOF-Cter and OTOF-Nter
- Assumption: ratio in samples is between 1/5 and 5
- Analysis of 3 independently prepared sets of 5 QC levels with 1:5 of 5:1 Cter-Nter ratio
    - Cter: CV% < 13%, RE% < 33%
    - Nter: CV% < 29%, RE% < 37%

Recovery

- Evaluated for representative or target tissues, feces and biological fluids. Includes PCR inhibition assessment
- At least one set of samples per extraction kit
- Spiking of vectors in sample homogenates (from 5 individuals)
- Validated for nasal & ear swabs, CSF, Urine
- 50% in temporal bone & auditory nerve. 20% for feces

Stability (on going)

- Evaluated for liver or brain, auditory nerve and whole eye, blood, temporal bone, feces, and fluids samples (i.e., CSF, ear swab, nasal swab, and urine samples)
- High and low levels assessed, Freeze and thaw as well as storage duration at -80°C to cover sample storage duration

Conclusion

The duplex qPCR assay for the determination of both analytes in NHP tissues, feces and fluids displays adequate performance to be used for biodistribution studies in NHP

Sample analysis is on going

- 22 tissues, blood, urine, feces, saliva, tears, nasal swabs, lacrimal swabs & CSF: around 2000 samples
- Expression of the results:
    - Copy number per µg of DNA in tissues and blood
    - Copy number per volume of fluid
    - Copy number per sample (swab)

Perspectives

- Long term stability assessment
- Validation of the RT-qPCR assay on going
    - Testing of tissues positive for the presence of test item DNA
- Validation of the duplex qPCR assay in mouse tissues

Thanks

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- Analysts: Quentin Henri, Océane Payet, Candyce Roland, Thommy Brisseau
- Léa Bauduin, Mylène Marette and Olivier Mégret, managers of the Molecular biology laboratory

Sensorion

- Nitza Thomasson, Study Monitor, NTZ consulting
- Laurent Désiré, Head of Preclinical Development, Sensorion

Questions ?