Development of an Immunocapture LC/MS Assay for Transgene Expression Quantification Using Commercially Available Anti-protein Antibody to Support Multi-species Gene Therapy Program

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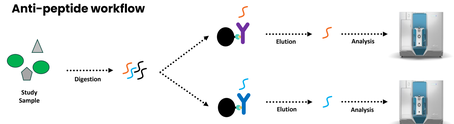
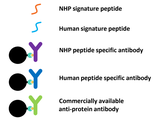
PURPOSE

- Anti-peptide antibodies are a bedrock standard for measuring transgene expression using signature peptide LC/MS analysis.
- Highly specific anti-peptide antibodies can be costly, time consuming to acquire, and may require additional species-specific anti-peptide antibodies when switching from preclinical species to humans.
- Here we describe a case study using a more conventional **anti-protein** antibody workflow which allows for rapid transitioning of species-specific analysis by switching signature peptides without generating additional species-specific anti-peptide antibodies.

Anti-peptide workflow



Anti-protein workflow



METHOD

- Three commercially available antibodies targeting different epitopes were evaluated.
- Antibody titration was performed to optimize antibody concentration for maximum recovery.
- Unique signature peptides from the transgene protein were identified for NHP and human.
- For the human assay, the high degree of homology between the endogenous wild-type protein and the transgene protein imposed a significant challenge for signature peptide selection. The signature peptide selected for quantification in human matrix contains the fusion point to differentiate transgene protein from wild-type protein.

RESULTS

Signature Peptides and Selectivity in NHP and Human Serum

Table 1. Signature Peptides Comparison between Transgene Product and Wild-type Counterparts

	Transgene	NHP Wild-type	Human Wild-type
Signature peptide NHP Assay	GDAVXXXXXRPXXXXXX	GDSIXXXXXVIPXXXXXX	GDAVXXXXXRPXXXXXX
Signature peptide Human Assay	STLKPXXXXXX	CSLRPXXXXXX	CTLKPXXXXXX

Figure 1. S-peptide Selectivity in Human Serum

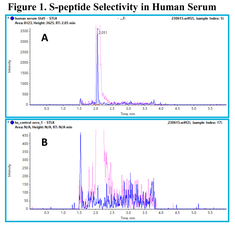


Figure 2. G-peptide Selectivity in NHP Serum

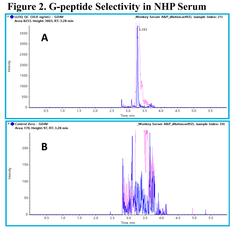


Table 1 demonstrates the signature peptide sequence difference between the transgene product and the wild-type counterparts in either NHP or human matrix. G-peptide was selected for NHP assay due to its high sensitivity and selectivity in NHP matrix. However, the exact sequence exists in the human wild-type protein rendering this peptide unusable for the human assay. An alternative peptide, S-peptide, was used for the human assay. The cysteine in wild-type protein was replaced by serine in the transgene product. With this one amino acid difference, we were able to differentiate and quantify the transgene product in the human matrix.

Fig. 1A shows a chromatogram peak of S-peptide at 50 ng/mL of transgene product in human serum; **Fig. 1B** shows a chromatogram of blank human serum indicating no interference at the retention time of the S-peptide. The pink trace is stable isotope labeled internal standard.

Fig. 2A shows a chromatogram peak of G-peptide at 50 ng/mL of transgene product in NHP serum; **Fig. 2B** shows a chromatogram of blank NHP serum indicating no interference at the retention time of the G-peptide. The pink trace is stable isotope labeled internal standard.

Antibody Titration

Figure 3. Capture Anti-protein Antibody Concentration Relation to Transgene Product Recovery

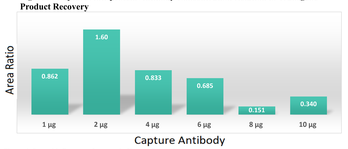


Figure 4. S-peptide Response Increase in Human Serum by Reducing Capture Ab Concentration

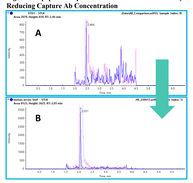


Figure 5. G-peptide Response Increase in NHP Serum by Reducing Capture Ab Concentration

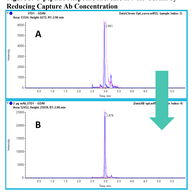


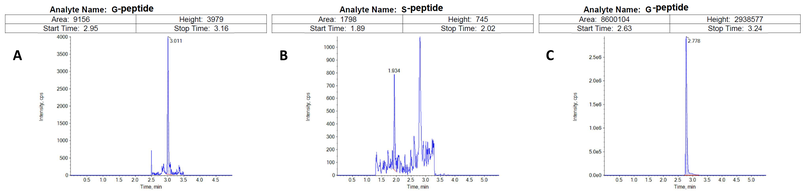
Fig. 3 demonstrates capture antibody titration results. Considering the presence of a high concentration of wild-type protein in the matrix, very high antibody-to-transgene protein mol ratios were evaluated. Surprisingly, high capture antibody performed poorly compared to low antibody capture. The antibody quantity for optimal capture recovery is 1-2µg.

Fig. 4A shows a chromatogram peak of S-peptide at 50 ng/mL of transgene product in human serum when captured using 10µg anti-protein capture antibody; **Fig. 4B** shows the same experiment but captured using 2µg antibody. The peak response increased about 5-fold when antibody was decreased from 10µg to 2µg.

Fig. 5A shows a chromatogram peak of G-peptide at 50 ng/mL of transgene product in NHP serum when captured using 10µg anti-protein capture antibody; **Fig. 5B** shows the same experiment but captured using 2µg antibody. The peak response increased about 5-fold when antibody was decreased from 10µg to 2µg.

Representative Quantification Results in NHP and Human Serum

Figure 6. Representative Chromatograms at LLOQ (5.00 ng/mL) in NHP and Human Serum



The transgene product quantification calibration range was 5.00 – 1000 ng/mL. **Fig. 6A** shows a representative chromatogram of G-peptide in NHP serum at the LLOQ. **Fig. 6B** shows a representative chromatogram of S-peptide in human serum at the LLOQ. **Fig. 6C** shows the same human serum LLOQ sample where G-peptide was simultaneously monitored indicating the presence of a high level of endogenous human wild-type protein not interfering with the assay.

Table 2. Quality Control Sample Intraday Accuracy and Precision
Transgene Product Concentrations (ng/mL) in Human Serum

Analytical Run	Replicate	Reported Concentration (ng/mL) 5.00	15.0	150	750
AR01-HuSER	1	5.01	15.7	155	807
	2	6.56 [a]	14.6	154	724
	3	5.98	13.8	151	862
	4	5.91	16.5	157	817
	5	6.63 [a]	15.6	153	829
	6	5.39	15.7	153	829
	Mean	5.91	15.3	154	811
	%CV	10.8	6.25	1.33	5.75
	%DEV	18.2	2.00	2.67	8.13

a QC sample calculates outside twenty five percent deviation from LLOQ

Table 3. Quality Control Sample Intraday Accuracy and Precision
Transgene Product Concentrations (ng/mL) in Cynomolgus Monkey Serum

Analytical Run	Replicate	Reported Concentration (ng/mL) 5.00	15.0	150	750
AR01-CynoSER	1	5.80	17.2	171	788
	2	5.99	16.4	158	794
	3	6.07	12.3	173	811
	4	5.98	16.5	172	783
	5	5.32	16.4	164	743
	6	5.28	15.3	166	758
	Mean	5.74	15.7	167	780
	%CV	6.14	11.3	3.46	3.18
	%DEV	14.8	4.67	11.3	4.00

CONCLUSION

- When anti-peptide antibodies are not readily available, anti-protein antibodies can serve as a viable option for transgene protein expression measurement to support gene therapy programs.
- The appropriate use of anti-protein antibodies for capture should be carefully evaluated to determine if the affinity of the antibody for endogenous protein components will impact method performance.
- The concentration of anti-protein capture antibody should be optimized to reach maximum capture efficiency of the transgene protein and immunocapture should be paired with a highly selective quantification method by identifying a transgene-specific signature peptide to distinguish the desired analyte from the potential interference of an endogenous component.
- Here we describe the development and use of an immunoaffinity capture LC/MS method with signature peptide analysis that allowed for rapid data generation without sacrificing accuracy, precision, or reproducibility. Flexibility for analysis across multiple species was streamlined via switching the selective identification of appropriate signature peptides for each species, eliminating the need to produce additional anti-peptide antibodies or other species-specific reagents when transitioning from pre-clinical to clinical studies to support this gene therapy program.