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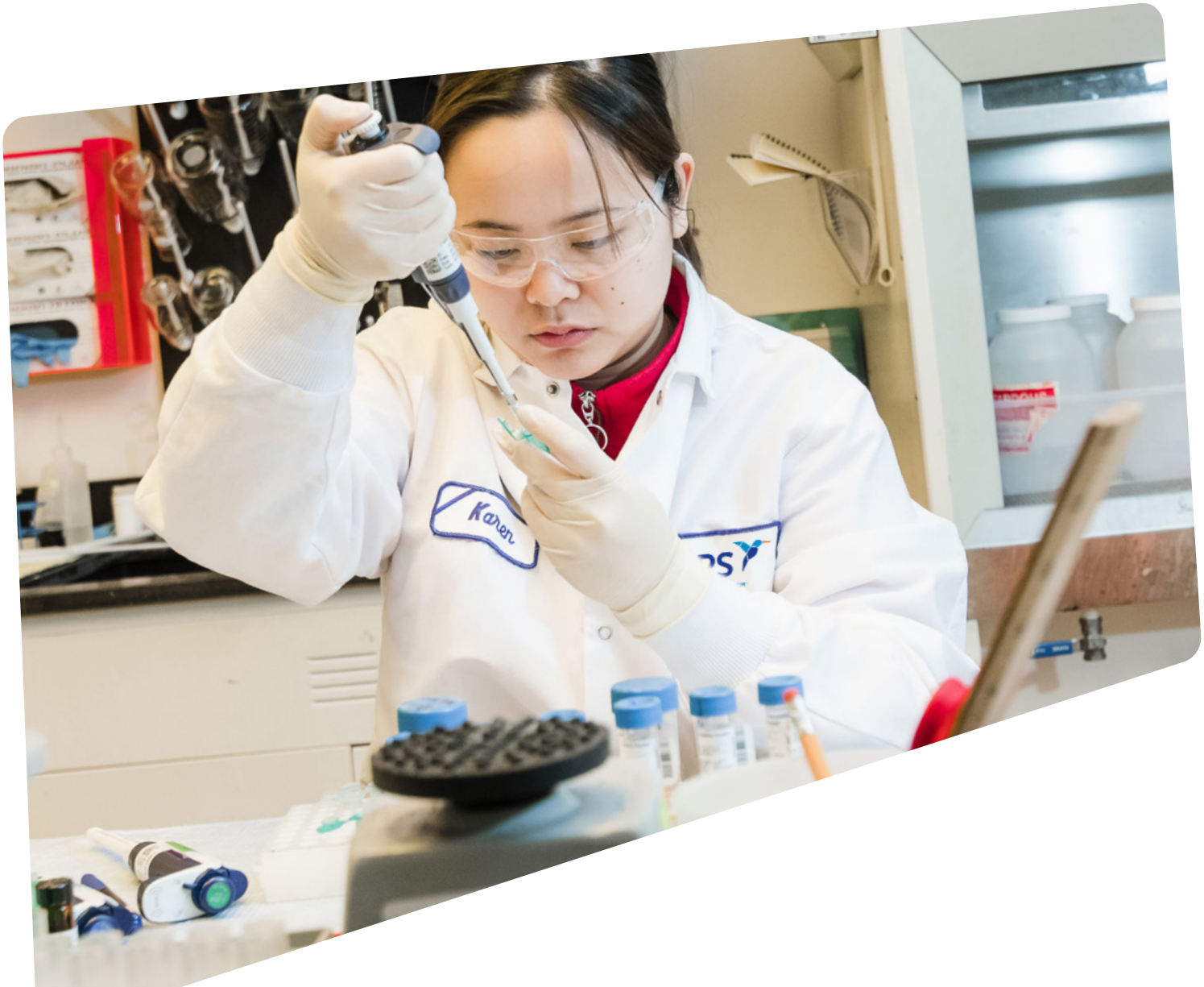
HYBRID LC-MS/MS METHODS FOR PK AND BIOMARKER ASSAYS IN **DRUG DEVELOPMENT STUDIES**



ASSAY VALIDATION ACCORDING TO LIGAND BINDING ASSAYS CRITERIA AND CHROMATOGRAPHIC METHODS

Strengthened by a dedicated team of biochemists and cell biologists trained in protein mass spectrometry, QPS has developed several hybrid LC-MS/MS methods using multiple reaction monitoring (MRM) for quantitation of biologics, including therapeutic antibodies, recombinant proteins, and peptides.

Superior in specificity, selectivity, and multiplexing ability, when used together with (immuno) affinity purification in hybrid methods, LC-MS/MS can deliver the sensitivity, throughput, and miniaturization provided by modern ligand binding assays (LBA). The resulting set-up allows both functional knowledge with high molecular detail, providing more accurate insights into pharmacokinetic and metabolism of biotherapeutics (Figure 1).

During (early) preclinical drug development, from lead optimization, candidate selection, and toxicity studies, hybrid LC-MS/MS methods offer time-effective and versatile bioanalytical solutions, bypassing the need for high-quality custom reagents to produce reliable PK profiling. If appropriately designed, a single hybrid LC-MS/MS assay has the potential to accompany biotherapeutics development up to clinical investigation, from exploratory to compliant bioanalytical studies.

KEY CRITERIA FOR DEFINING THE BIOANALYTICAL STRATEGY

LBA, in particular enzyme-linked immunosorbent assays (ELISA), have long been the standard method for PK measurements of therapeutic antibodies, because of their high sensitivity and throughput. However, beside depending on reagents availability, LBA often lack the selectivity needed to distinguish between significant molecular differences (such as point mutations in the target protein or post-translational modifications) and require the implementation of dedicated platforms for multiplexing.

The extent of anticipated cross-reactivity, the required dynamic range, robustness, and throughput, drive the bioanalytical strategy during drug development.

Selecting the appropriate platform depends on the stage of drug development (exploratory, preclinical, clinical), selectivity and sensitivity requirements, the expected concentration range, the availability of well-defined reagents and of a representative internal standard. Equally important, timelines and regulatory aspects influence the final decision for choosing one or more bioanalytical platform(s).

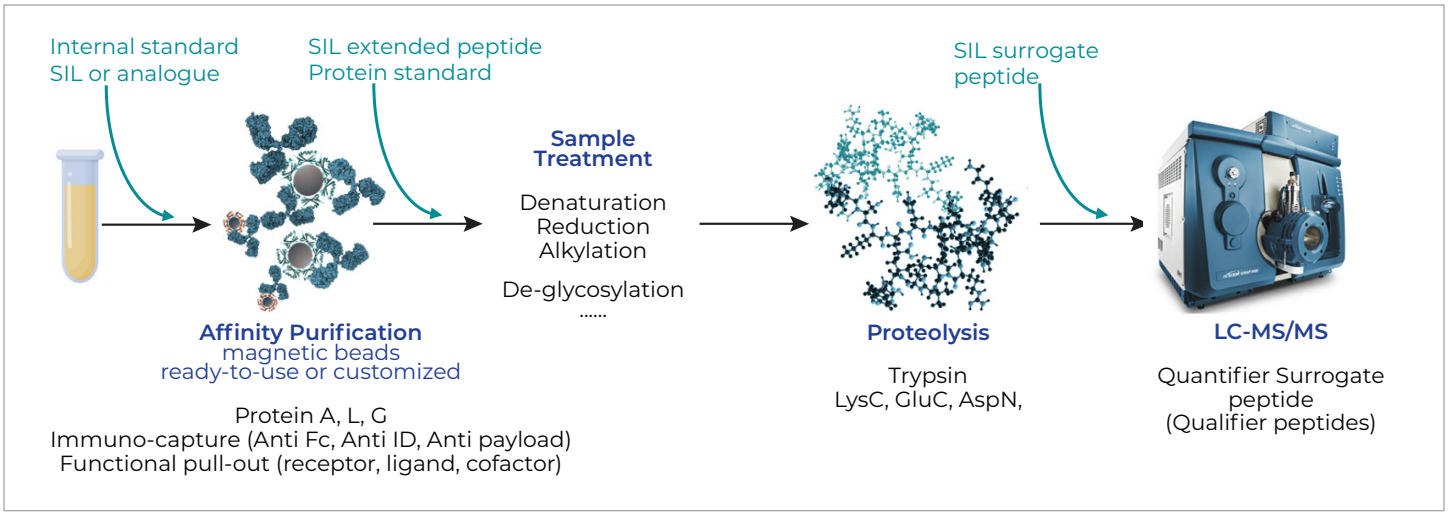


Figure 1: General experimental setup. Absolute and regulatory compliant quantitative data is acquired by monitoring the intensity response from signature/surrogate peptides obtained through enzymatic digestions of the target protein. As demonstrated for standard chromatographic bioanalytical methods, addition of an internal standard (IS) displaying similar or identical physiochemical properties, is essential for minimizing technical variations during sample preparation and detection. Further purification of the digested sample by Solid Phase Extraction (SPE) preceding chromatography may be of help for achieving higher sensitivity and improve assay performance.

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GENERIC AND MULTIPLEXING ASSAYS FOR THERAPEUTIC ANTIBODIES

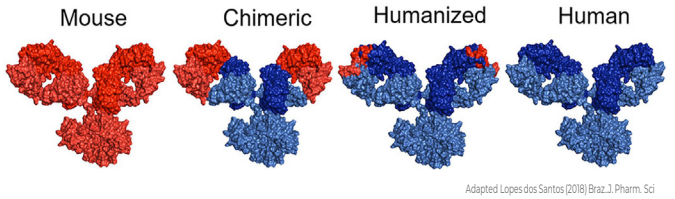
QPS offers GLP validated hybrid LC-MS/MS assays for PK evaluation of humanized therapeutic antibodies in rat and monkey serum for preclinical development, which build on the general experimental set-up, and monitor surrogate peptides mapping in the conserved regions of the immunoglobulin backbone (Figure 2).

These generic methods are ready-to-use for any preclinical development programs involving humanized IgG-based drugs and are customizable for increased selectivity to include specific peptides from the complementarity-determining regions (CDR) or other domains for monitoring stability, or collect catabolic information (Time frame: method development/method validation, 3-4 weeks).

Molecule-specific quantitative methods allow multiplexing for simultaneous quantification of two or more co-administered mAbs or other biologics, i.e., exploratory cassette-dosing studies as well as in late phase combination therapy investigational studies (Figure 3).

SPECIFIC HYBRID LC-MS/MS METHODS FOR PK EVALUATION OF RECOMBINANT PROTEINS

Bioanalysis of therapeutic recombinant or transgenic proteins by LBA poses a greater challenge due to the potential higher level of cross-reactivity with endogenous copies. A hybrid LC-MS/MS assay can provide a valuable solution if selective surrogate peptides can be obtained from proteolysis of the therapeutic and endogenous version(s) of the target protein. Such PK assays can be implemented to support replacement (genetic or enzyme or hormone) therapy programs. The hybrid

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mAb1 (IgG4)  SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
mAb2 (IgG1)  SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
mAb3 (IgG1)  SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
mAb4 (IgG1)  SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
                                   generic

mAb1 (IgG4)  KASGITFSNSGMHWVRQAPGKGLEWVAVIWYDGSKRYYADSVKGRFTISRDNSKNTLF
mAb2 (IgG1)  AASGFNIKDTYIHWVRQAPGKGLEWVARIYPNGYTRYADSVKGRFTISADTSKNTAYL
mAb3 (IgG1)  AASGFTFTDYTMDWVRQAPGKGLEWVADVNPSGGSIYNQRFKGRFTLSVDRSKNTLYL
mAb4 (IgG1)  AASGYTFTNYGMNWVRQAPGKGLEWVGWINTTGEPTYAADFKRRFTFSLDTSKSTAYL
                                   mAb-specific
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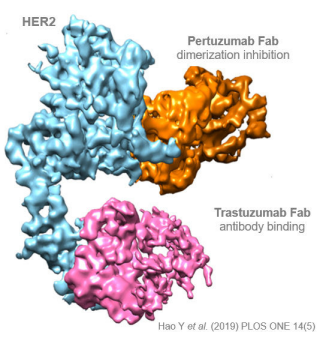
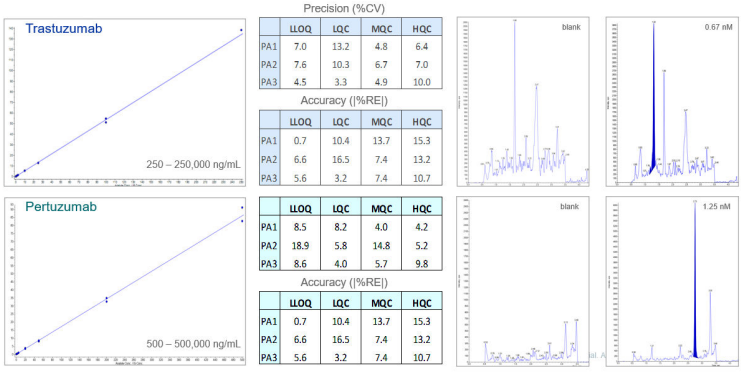
Figure 2 (left): Generic and specific hybrid LC-MS/MS assays for bioanalysis of therapeutics mAbs. Surrogate peptide selection is key for method development. Generic methods monitor unique signature peptides mapping in the conserved domains and can be readily applied for bioanalysis of the same IgG isotypes. For the development of molecule-specific methods, surrogate peptides are selected in the variable regions (one amino acid substitution is often enough to confer sufficient selectivity).

Figure 3 (below): Nanomolar concentrations of trastuzumab and pertuzumab were determined in 10 µL serum samples after extraction by affinity purification through protein-A coated magnetic beads, followed by reduction, alkylation, and trypsin digestion. Quantitation was obtained by LC-MS/MS using SILuMab as internal standard. Two surrogate peptides mapping in the CDR regions allowed for simultaneous monitoring of both analytes. Linearity was established in the ranges 0.250-250 µg/mL for trastuzumab and 0.500-500 µg/mL for pertuzumab. The method was validated according to the current FDA/EMA guidelines for large molecule PK assays.

A complete description of the method is described in: Schokker, S, Fusetti, F, Bonardi, F, Molenaar, RJ, Mathôt, RAA, van Laarhoven, HWM, (2020) Development and validation of an LC-MS/MS method for simultaneous quantification of co-administered trastuzumab and pertuzumab. mAbs 12 (1), 1795492.



- Trastuzumab and pertuzumab used in combination in the clinic for the treatment in metastatic tumors
- Bind to different domains of the HER2 receptor
- mAbs cannot be simultaneously quantified with one ligand binding assay

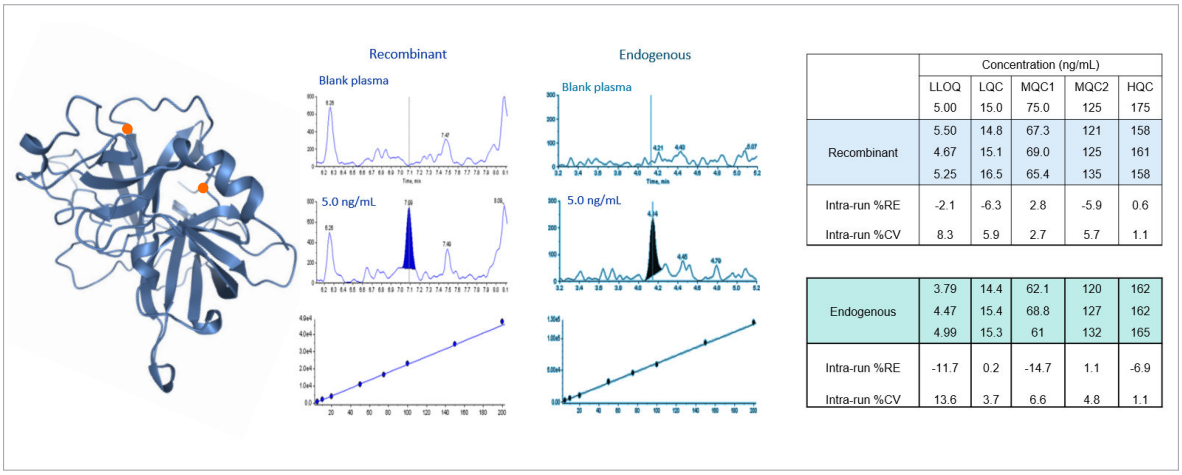


	LLOQ	LQC	MQC	HQC
PA1	7.0	13.2	4.8	6.4
PA2	7.6	10.3	6.7	7.0
PA3	4.5	3.3	4.9	10.0

	LLOQ	LQC	MQC	HQC
PA1	0.7	10.4	13.7	15.3
PA2	6.6	16.5	7.4	13.2
PA3	5.6	3.2	7.4	10.7

	LLOQ	LQC	MQC	HQC
PA1	8.5	8.2	4.0	4.2
PA2	18.9	5.8	14.8	5.2
PA3	8.6	4.0	5.7	9.8

	LLOQ	LQC	MQC	HQC
PA1	0.7	10.4	13.7	15.3
PA2	6.6	16.5	7.4	13.2
PA3	5.6	3.2	7.4	10.7



	Concentration (ng/mL)				
	LLOQ	LQC	MQC1	MQC2	HQC
	5.00	15.0	75.0	125	175
Recombinant	5.50	14.8	67.3	121	158
	4.67	15.1	69.0	125	161
	5.25	16.5	65.4	135	158
Intra-run %RE	-2.1	-6.3	2.8	-5.9	0.6
Intra-run %CV	8.3	5.9	2.7	5.7	1.1
Endogenous	3.79	14.4	62.1	120	162
	4.47	15.4	68.8	127	162
	4.99	15.3	61	132	165
Intra-run %RE	-11.7	0.2	-14.7	1.1	-6.9
Intra-run %CV	13.6	3.7	6.6	4.8	1.1

Figure 4: Development and quantitation of a hybrid LC-MS/MS method for the determination of a recombinant therapeutic protein. Two point mutations differentiate the recombinant from the endogenous protein (in red). A sensitivity of 75 pM was achieved by combining enrichment by immunoaffinity with monitoring of two specific and selective tryptic surrogate peptides, mapping in proximity of the mutated amino acid residues. Synthetic stable-labeled peptides were included as internal standard after trypsin digestion. Linearity was established in the range 5.0-200 ng/mL.

LC-MS/MS method summarized in Figure 4, is based on the general experimental set-up, and relies on a specific immunoaffinity extraction of the target protein followed by tryptic digestion. Because of its high selectivity these methods can be seamlessly transferred across matrices, including tissue homogenates, and are suitable for bioanalysis of preclinical to clinical study samples. (Time frame: method development/method validation 4-6 weeks).

Applications:

- Monoclonal therapeutic antibodies
- Antibody-Drug-Conjugates
- Recombinant proteins in transgenic and genetic therapies
- Therapeutic peptides
- Peptide-drug conjugates

Dedicated Equipment:

- SCIEX Triple Quad™ 6500/6500+
- Shimadzu Nexera/Nexera2 and Agilent 1290 Infinity UPLC
- KingFisher™ Flex purification system for automated handling of magnetic beads and protein digestion
- Versette (Thermo) for automated immunocapture and customized affinity purification
- Tomtec Quadra 96 Liquid Handling platform

WHY QPS?

- We have built up vast experience since 2016 developing and validating hybrid LC-MS/MS methods for the quantitation of many biotherapeutics in various animal species and matrices.
- We have fully GLP compliant laboratories in USA and Europe.
- We have 5 dedicated hybrid LC-MS/MS systems and thus sufficient capacity for supporting large (preclinical and clinical) studies.
- We are very flexible, cooperative and have a broad experience in biochemistry, enzymology, molecular biology, mass spectrometry, and bioanalytical chemistry, including regulatory aspects.
- We are used to conducting complex studies.
- We value face-to-face meetings. You are welcome to visit us and view our laboratories.

Whether your focus is:

- Small molecules
- Protein biotherapeutics
- Vaccines
- Gene therapy

QPS provides a full range of bioanalytical solutions to support drug development from discovery through clinical development and filing.

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Time is of the essence in drug development.
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