Method Development Guidelines

Primary structure analysis methods

This section on column selection strategy for primary structure analysis provides some critical details on method development for mAb, proteins, and peptides.

Peptides and polypeptides Mol Wt <10 kDa	Proteins Mol Wt >10 kDa	Reduced/fragmented Mol Wt <50 kDa	Intact Mol Wt >150 kDa
Initial bonded phase			
AdvanceBio Peptide Mapping Also consider: ZORBAX 300 Å StableBond C18	**ZORBAX 300 Å StableBond C8** Also consider: PLRP-S ZORBAX 300 Å StableBond C18 ZORBAX 300 Å StableBond C3 Poroshell 300 StableBond C18 Poroshell 300 StableBond C8 Poroshell 300 StableBond C3	**AdvanceBio RP-mAb StableBond C8** Also consider: PLRP-S AdvanceBio RP-mAb C4 AdvanceBio RP-mAb diphenyl ZORBAX 300 Å diphenyl ZORBAX 300 Å StableBond C8 ZORBAX 300 Å StableBond C3 ZORBAX 300 Å StableBond C18	**AdvanceBio RP-mAb C4** Also consider: PLRP-S AdvanceBio RP-mAb StableBond C8 AdvanceBio RP-mAb diphenyl Poroshell 300 StableBond C3 Poroshell 300 StableBond C8
Initial separation conditions			
Mobile phase: A: 95% H_2O:5% ACN with 0.1% TFA B: 5% H_2O:95% ACN with 0.085% TFA **Gradient:** 3 to 60% in 30 min **Temperature:** 40 °C	**Mobile phase:** A: 95% H_2O:5% ACN with 0.1% TFA B: 5% H_2O:95% ACN with 0.085% TFA **Gradient:** 5 to 70% in 20 min **Temperature:** 80 °C	**Mobile phase:** A: 100% H_2O with 0.1% TFA B: 10% H_2O:10% ACN:80% n-PA with 0.08% TFA **Gradient:** 5 to 40% in 10 min **Temperature:** 60 °C	**Mobile phase:** A: 98% H_2O:2% IPA with 0.1% TFA B: 10% H_2O:0% ACN:70% IPA with 0.08% TFA **Gradient:** 10 to 60% B in 5 min **Temperature:** 80 °C
2.1 mm id columns **Particle size** 2.7 µm SPP **Flow rate** 0.5 mL/min	**4.6 mm id columns** **Particle size** 2.7 µm SPP **Flow rate** 2.0 mL/min	**2.1 mm id columns** **Particle size** 1.8 µm TPP 3.5 µm TPP 5 µm TPP 5 µm SPP **Flow rate** 0.5 mL/min 0.2 mL/min 0.2 mL/min 1.0 mL/min	**4.6 mm id columns** **Particle size** 3.5 µm TPP 5 µm TPP **Flow rate** 1.0 mL/min 1.0 mL/min	**2.1 mm id columns** **Particle size** 3.5 µm SPP 1.8 µm TPP 3.5 µm TPP 5 µm TPP **Flow rate** 0.8 mL/min 0.3 mL/min 0.2 mL/min 0.2 mL/min	**4.6 mm id columns** **Particle size** 3.5 µm SPP 3.5 µm TPP 5 µm TPP **Flow rate** 4.0 mL/min 1.0 mL/min 1.0 mL/min	**2.1 mm id columns** **Particle size** 3.5 µm SPP 5 µm SPP **Flow rate** 1.0 mL/min	**4.6 mm id columns** **Particle size** 3.5 µm SPP **Flow rate** 5.0 mL/min

SPP = superficially porous particle, TPP = totally porous particle

Start at low pH with simple aqueous/organic gradient

Typically, a water:acetonitrile with 0.1% trifluoroacetic acid (TFA) gradient is used to elute all components of interest. A typical high resolution gradient on a 300 Å pore size column requires 30 to 50 min. An AdvanceBio RP-mAb column requires a shorter analysis time and a higher flow rate, and still provides exceptional resolution. To improve resolution, increase the gradient time, decrease column length, or increase flow rate. For LC/MS methods, TFA can reduce detector sensitivity and is often replaced with ammonium formate/formic acid.

Optimize sample solubility

For best peak shape and recovery at any pH, it is important to completely solubilize a sample. Highly acidic or neutral solvents can be used with AdvanceBio RP-mAb, ZORBAX 300 Å StableBond, Poroshell 300 StableBond, and AdvanceBio Peptide Mapping, while neutral solvents and dilute bases can be used with ZORBAX 300Extend-C18 and Poroshell 300Extend-C18.

Solvent choices to solubilize proteins and peptides

Weakest

- Water/phosphate buffer
- Dilute acid (TFA, acetic acid or HCl)
- Neutral pH, 6–8 M guanidine-HCl or isthiocyanate
- Acetic acid 5%/6 M urea
- Dilute acid + aqueous/organic solvents (ACE, MeOH, THF)
- Dilute base (ammonium hydroxide)
- DMSO or 0.1%–1% in DMSO
- Formamide

Strongest

Increase the temperature

Separations of proteins and peptides are influenced by temperature and higher column temperature can dramatically improve both resolution and recovery of proteins and hydrophobic and aggregating peptides.

AdvanceBio RP-mAb: Up to 90 °C
ZORBAX 300 StableBond, Poroshell 300 StableBond: Up to 80 °C
AdvanceBio Peptide Mapping: Up to 60 °C

Optimize mobile phase pH

Try mid and high pH if low pH does not work

If an optimized, low pH method does not provide an ideal separation, then mid or high pH mobile phase can be used. At high pH, selectivity is often very different because acidic amino acids become negatively charged and some basic amino acids may lose their charge. ZORBAX 300Extend-C18 is an excellent choice for mid to high pH separation.

Column:	**ZORBAX 300Extend-C18 4.6 x 150 mm, 5 µm 773995-902**	Gradient:	5–60% B in 30 min
Mobile phase:	A: 20 mM nH_4OH in H_2O B: 20 mM nH_4OH in 80% ACN	Temperature:	25–30 °C (<60 °C)
		Flow rate:	1 mL/min