Guideline for the enrichment of proteins from FFE-fractions

Introduction

The pre-fractionation of complex protein mixtures using free flow electrophoresis provides an enormous potential to increase sensitivity and efficiency of protein analysis. The method can be applied to a variety of samples and is compatible with the most widely used downstream analysis methods such as 2D PAGE and chromatography based techniques.

The appropriate method for sample preparation after free flow electrophoresis is an important step to take fully advantage of the FFE technique. This guideline offers hints and recommendations of methods that appear to be most suitable for the various analysis techniques.

Overview

	Analytical post-FFE-technique	Potentially interfering additives	Possible concentration / clean up step	Comment	Applicable FFE-protocol
Analysis of native proteins	**FFE-Gel-MS**	None	UF* or SPE*	direct application of FFE-fractions OR concentration up to 10-fold	all for SPE or uncleaned fractions OR low HGP for UF
	FFE-MS	HPMC, glycerol, Prolytes	SPE	removal of HPMC, glycerol, Prolytes	all
	FFE-Crystallization	HPMC, glycerol, Prolytes	SPE	removal of HPMC, glycerol, Prolytes	all
	FFE-Digestion-LC-MS	HPMC, glycerol, Prolytes	UF	concentration up to 10-fold (removal of HPMC from peptides with UF)	low HGP
			SPE	removal of HPMC, glycerol, Prolytes	all
	FFE-LC-MS	HPMC, glycerol	SPE	removal of HPMC and glycerol	all
Analysis of denatured proteins	**FFE-Gel-MS**	none	UF or SPE	direct application of FFE-fractions OR concentration up to 10-fold	all for SPE or uncleaned fractions OR low UHGP for UF
	FFE-MS	HPMC, glycerol, Prolytes, urea	SPE	removal of HPMC, glycerol, Prolytes, urea	all
	FFE-Digestion-LC-MS	HPMC, glycerol, Prolytes, (urea)	UF	concentration up to 10-fold (removal of HPMC from peptides with UF)	low UHGP
			SPE	removal of HPMC, glycerol, Prolytes, urea	all
	FFE-LC-MS	HPMC, glycerol	SPE	removal of HPMC and glycerol	all

* UF ...Ultrafiltration
* SPE ...Solid Phase Extraction

(1) ULTRAFILTRATION of FFE fractions

Centrifugal ultrafiltration is a separation process, that uses anisotropic semipermeable synthetic membranes to concentrate or separate biopolymers via centrifugation based on their size. There exist quite a number of different suppliers of these devices, but it is suggested to use “Vivaspin” ultrafiltration devices (Vivascience, Germany; http://www.vivascience.com) because of some very specific features:

The patented vertical membrane design and thin channel filtration chambers minimise membrane fouling and provide high speed concentrations, even with particle laden solutions, and excellent recovery rates. The membranes with the best performance are made from polyethersulfone and are available with different molecular weight cut-offs (5 kDa, 10 kDA, 30kDa, 50 kDa and 100 kDa), but in the FFE context it is suggested to use the 5 kDa membranes.

There exist several sizes for fraction volumes of up to 500µL (VIVASPIN 500), 2mL (VIVSPIN2), 4mL (VIVSPIN4), 6mL (VIVSPIN6), and 15mL (VIVSPIN15). In principle, all FFE protocols can be used in combination with ultrafiltration, but the more HPMC the media contains, the lower the concentration factor that can be reached, because HPMC won’t pass the filter membrane and will get concentrated, too.

This means that the protocols with the prefix “low” will work better than the ones with the prefix “high”, because this indicates the relative amount of HPMC used for each protocol. The “low UHGP” protocol included in the manual works best and will allow concentration factors of approximately 10.

The fractions are just poured into the devices and centrifuged according to the manufacturer’s instructions until the desired (or maximal) concentration is reached. It might be useful to shift the pH of the fractions prior to ultrafiltration (especially in the case of “native” separation media, because otherwise the proteins might stick to the membranes based on their uncharged state close to their pI.

After centrifugation, the remaining fractions are pipetted out of the devices by means of a normal pipette and immediately used for subsequent gel-based analysis. The Vivaspin devices were tested for the enrichment of (membrane-)proteins from urea/thiourea containing FFE-fractions, which were obtained from raw homogenates of rat kidney, rat liver, *E. coli*, yeast mitochondria and rat microsomal fractions. Generally, the approach is meant to be done manually with fractions of particular interest, due to the absence of a 96-well format device.

(2) Solid Phase Extraction of FFE fractionsc (Hydrophilic interaction chromatography) Materials:

• Isopropanol (p.a. or HPLC grade)
• Hexafluoro-isopropanol (HFIP)
• Sodiumchloride (NaCl, 1M solution in HPLC grade water)
• Formic acid (p.a., concentrated, 98%)
• Poly-Hydroxyethylaspartamide (HEA): Silica beads coupled (12 µm diameter, 300angström pore size))
• Preferably ready to use SPE plate like available from: PolyLC US (part number SPEHY1203-96/70)
• Vacuum apparatus for SPE plate processing (manual device or Te-VacS module for robotic processing on a Genesis or Freedom platform)

Method (manual operation)

1. Typical FFE experiment: Run FFE in IEF mode and collect fractions (1ml size) in Minititer plates (removable, 1ml tubes)
2. Total volume collected may range from 1ml to 10ml
3. Dilute FFE fraction in **tenfold** excess of *binding buffer* (90% isopropanol, 10mM NaCl, 100mM HFIP, 200mM formic acid)
E.g. 1ml FFE makes up to 10ml total volume.
4. Apply FFE fraction(s) diluted in binding buffer to SPE plate
5. Allow binding of proteins to the resin by applying vacuum (200-500 mbar)
A typical filtration step takes 20 minutes for 10ml of sample applied to each well
6. Wash resin with 1ml binding buffer
7. Wash resin with 2 x 1ml of 90% isopropanol only – removal of additives from previous step
8. Apply vacuum until resin is quasi dry
9. Apply elution buffer, typically 3 x 100µl (i.e. 300µl total)
must not contain excess organic solvent, especially little isopropanol
composition of elution buffer may be dependant on the nature of downstream analytics, e.g.:
• Chromatographic separation of FFE fractions – use HPLC starting buffer (w. low organic solvent)
• 2D gel electrophoresis – use 2DE sample buffer (9M urea, etc.)
10. Collect eluted sample
11. Proceed w. downstream analytics

Please note:

1. Plugging of plate may occur after approx. 15ml esp. in acidic – try to break down larger experiments into 10ml pieces and process one after another.
2. 70 mg HEA elute best w. 3 x 100µl buffer, hence 300µl is the minimal recovery volume!
3. Concentration of isopropanol is crucial and needs to be 90% !