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# Extraction of Vitamin B7 (Biotin) from Serum Using EVOLUTE<sup>®</sup> EXPRESS ABN Prior to LC-MS/MS Analysis

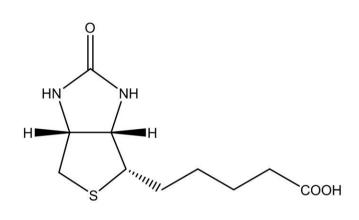


Figure 1. Structure of Vitamin B7.

#### Introduction

Vitamin B7 (Biotin) is a water soluble vitamin necessary for cell growth. The method described in this application note achieves high reproducible extraction recoveries of vitamin B7 from serum while minimizing co-extractable material in the form of proteins, lipids and phospholipids. Serum is extracted using the EVOLUTE® EXPRESS ABN 96-well plate.

EVOLUTE EXPRESS SPE products dramatically improve flow characteristics, and enhance sample preparation productivity providing clean, robust, sample preparation. This method can be automated using Biotage<sup>®</sup> Extrahera<sup>™</sup> (see appendix for details).

#### Analytes

Biotin (with Biotin-[<sup>2</sup>H<sub>2</sub>] as internal standard).

#### Sample Preparation Procedure

#### Format

EVOLUTE® EXPRESS ABN 10 mg plate, part number 600-0010-PX01.

#### Sample Pretreatment

To 200  $\mu$ L of serum add internal standard (Biotin-[<sup>2</sup>H<sub>2</sub>]) at 250 pg/mL and dilute using with 1% formic acid (aq) (200  $\mu$ L). Mix.

#### **Condition (Optional)**

Condition each well with methanol (500 µL). This step is not required with the EVOLUTE EXPRESS Load-Wash-Elute procedure.

#### **Equilibration (Optional)**

Equilibrate each well with 1% formic acid (aq) (500 µL). This step is not required with the EVOLUTE EXPRESS Load-Wash-Elute procedure.

#### Sample Loading

Load 400 µL of pre-treated serum into each well.

#### Wash 1

Elute interferences with  $H_2O$  (500 µL).

#### Wash 2

Elute interferences with  $H_2O/MeOH$  (95/5, v/v, 500 µL).

#### Elution

Elute analytes with 0.1%  $\rm NH_4OH$  in (H\_2O/MeOH, 90/10, v/v, 200  $\mu L).$ 

#### **Post Elution**

Evaporate to dryness at 40 °C in a stream of air or nitrogen using a Biotage<sup>®</sup> SPE Dry.

#### Reconstitution

Reconstitute the extract with  $H_2O/ACN$  (90/10, v/v, 200 µL).



#### **UPLC Conditions**

#### Instrument

Waters ACQUITY I-Class

#### Column

ACE Excel 1.7 µ C18-PFP column (100 x 2.1 mm id)

#### **Mobile Phase**

A: 1 mM ammonium fluoride (aq).

B: Acetonitrile.

#### **Flow Rate**

o.4 mL/min.

Table 1. Gradient Conditions.

Time	% A	% B	Curve
0	90	10	1
1.50	82	18	6
2	20	80	1
2.6	90	10	1

Curve 6: Lineat Gradient

#### **Injection Volume**

10 µL

#### Sample Temperature

20 °C

#### **Column Temperature**

40 °C

#### Mass Spectrometry Conditions

#### Instrument

Xevo TQ-S triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

#### **Desolvation Temperature**

500 °C

#### **Ion Source Temperature**

150 °C

#### **Collision Cell Pressure Temperature**

3.7 e<sup>-3</sup> mbar

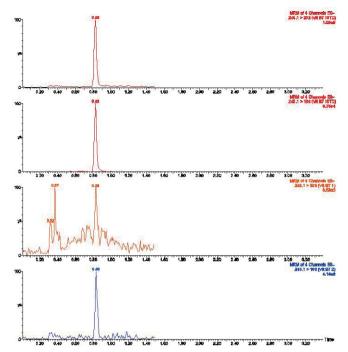
Negative ions acquired in multiple reaction monitoring (MRM) mode:

#### Table 2. MRM Conditions.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Vitamin B7 (Qual)	243.1 > 200.0	25	15
Vitamin B7 (Quant)	243.1 > 166.0	25	15
Vitamin B7-[ <sup>2</sup> H <sub>2</sub> ]	245.1 > 168.0	25	15

#### Results

Good retention and chromatographic peak shape was obtained using the C18-PFP column. Figure 2. demonstrates signal intensity and peak shape attained from serum spiked at 25 pg/mL with deuterated internal standard at 250 pg/mL.



**Figure 2.** Chromatography obtained from serum spiked at 25 pg/mL. Retention time for vitamin B7 (biotin) is approximately 0.8 mins.

#### Recovery

Stripped serum was spiked at various concentrations from 25–5000 pg/mL for recovery determination. High reproducible recoveries > 80% with corresponding RSDs < 10% were demonstrated. Typical recovery data for full and Load-Wash-Elute methods from spiked serum at 2000 pg/mL is shown in figure 3.

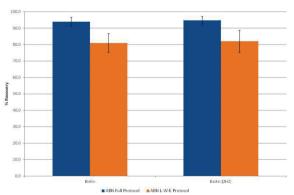


Figure 3. Spiked serum recovery profile for full and Load-Wash-Elute SPE protocols.



### **Calibration Curves**

Calibration curves were generated using stripped serum spiked at concentrations from 25–1000 pg/mL. Good linearity, coefficients of determination ( $r^2 > 0.99$ ) and sensitivity were obtained. Stripped serum matrix contained low residual endogenous levels of biotin which contributed to a slight intercept on the calibration curves. (see figure 4).

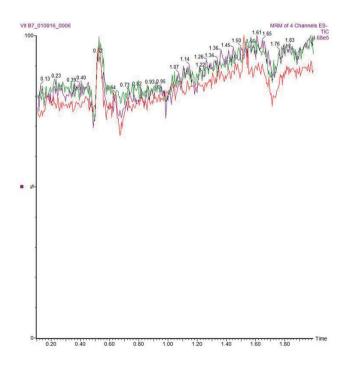
#### Extract Cleanliness

#### **Phospholipid Removal**

Post extraction residual phospholipid levels were investigated to provide an indication of extract cleanliness. The most abundant phospholipids in human serum (previously selected from full scan, SIR and precursor ion scanning experiments) were assessed using MRM transitions monitoring the common 184 product ion. Figure 5 demonstrates phospholipid content comparing 100  $\mu$ L of protein precipitated serum with the final EVOLUTE EXPRESS ABN extraction protocol using 200  $\mu$ L of matrix.

## Post Column Infusion

Extract cleanliness was also investigated using post-column infusion (PCI) experiments. Mobile phase and blank serum extracts were injected onto the LC-MS/MS setup while simultaneously infusing Vitamin B7, to determine regions of suppression, as shown in Figure 6.



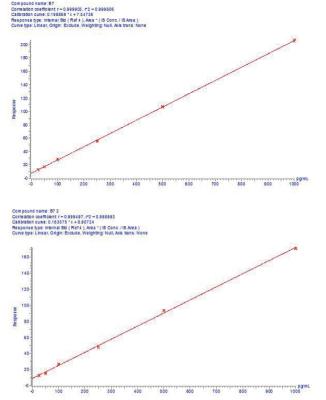


Figure 4. Serum quantifier and qualifier ion calibration curves spiked from 25–1000 pg/mL, extracted in duplicate.

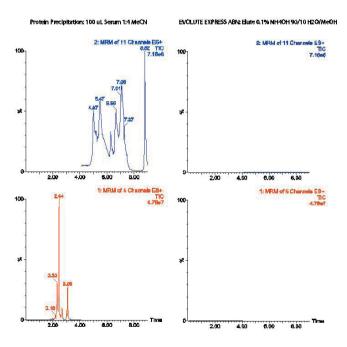


Figure 5. Phospholipid MRM TICs for final serum extraction protocol.

*ø* Biotage

Figure 6. PCI baselines comparing blank solvent (red) to extracted blank serum using full SPE (green) or L-W-E procedures (purple). Minimal baseline disturbance, indicating low matrix effects, is evident at  $\sim$ 0.8 minutes (analyte retention time).

## Additional Notes

#### **Reagent Preparation**

- 1% formic acid aq: Measure 99 mL of H<sub>2</sub>O and add 1 mL of formic acid (99% concentration).
- 2.  $H_2O/MeOH$  (95/5, v/v): Measure 95 mL of  $H_2O$  and add 5 mL of MeOH.
- 3. o.1% NH<sub>4</sub>OH in (90/10, v/v) H<sub>2</sub>O/MeOH: Measure 89.9 mL of H<sub>2</sub>O, add 100  $\mu$ L of NH<sub>4</sub>OH (aq, 28–32% concentration) followed by 10 mL of MeOH.
- 4.  $H_2O/MeCN$  (90/10, v/v): Measure 90 mL of  $H_2O$  and add 10 mL of MeCN.
- 5. 1 mM ammonium fluoride aq (mobile phase A): Weigh 0.03704 g and dissolve in H<sub>2</sub>O. Dilute and make up to 1 L in H<sub>2</sub>O.

#### **Mobile Phase and Ionization Considerations**

- As a small polar carboxylic acid, ionization of vitamin B7 is possible using both + and - ion modes.
- Negative ion mode provided better sensitivity due to the availability of more selective MRM transitions. Positive ion MRM showed a tendency for the water loss product ion [M+H-H<sub>2</sub>O]<sup>+</sup>.
- Acidic mobile phase additives are typically used to provide extra retention of acid moieties during chromatography. However, due to the use of negative ionization and optimization of signal to noise these additives were omitted.
- 4. Ammonium fluoride was selected to scavenge baseline noise in negative ion electrospray. This resulted in better signal to noise than other additive such as ammonium acetate or formate.
- 5. The  $NH_4F$  resulted in shorter retention of the target analyte due to increased pH.
- 6. MeCN was selected for the organic eluent as a polar aprotic option for negative ionization.

#### **SPE** Considerations

- EVOLUTE® EXPRESS ABN was compared to the mixed-mode strong and weak anion exchange sorbents. EVOLUTE EXPRESS AX also provided good recoveries and phospholipid removal. However, better cleanliness due to enhanced pigment removal was achieved using the optimized elution combinations with the ABN sorbent.
- pH control using acidic additives for sorbent conditioning (optional) and sample pre-treatment was aimed at suppressing ionization of the analyte making it less polar for the initial retention.
- 3. Washing steps were kept highly aqueous (to avoid losses due to analyte polarity) but were also intended to remove residual acidic nature to allow easier elution.
- 4. Final elution conditions were optimized for highly aq elution conditions which resulted in massively reduced phospholipid content in the extracts. Elution with  $H_2O/MeOH$  (50/50, v/v) demonstrated good removal of phospholipids but the addition of small amounts of base resulted in the analyte being more ionized, and therefore more polar, allowing elution using 90% aqueous conditions. This provided excellent extract cleanliness.
- 5. Minimum elution volume was optimized at 200  $\mu$ L for the highly aqueous elution solvent described above. A reduced volume (100–150  $\mu$ L) with an increased organic content could be used. However, this may adversely impact extract cleanliness, due to increased co-elution of matrix components.
- 6. It was not possible to eliminate evaporation and move to direct injection using the selected chromatographic mobile phases. The use of a pH stable column may allow direct injection. Alternatively, a  $H_2O/MeOH$  (50/50, v/v) elution solvent could be used with aq dilution prior to injection.

#### **Ordering Information**

Part Number	Description	Quantity
600-0010-PX01	EVOLUTE® EXPRESS ABN 10 mg Fixed Well Plate	1
For Manual Process	ing	
PPM-96	Biotage <sup>®</sup> PRESSURE+ 96 Positive Pressure Manifold	1
For Automated Proc	cessing	
414001	Biotage <sup>®</sup> Extrahera	1
415040	Configuration Kit 96 Positions Dual Flow	1
414141	Extrahera Clear Tips	960

Part Number	Description	Quantity
Accessories/Consu	mables	
121-5203	Collection Plate, 2 mL, Square	50
121-5204	Piercable Sealing Cap	50
Evaporation		
SD-9600-DHS-EU	Biotage <sup>®</sup> SPE Dry 96 Sample Evaporator 220/240V	1
SD-9600-DHS-NA	Biotage® SPE Dry 96 Sample Evaporator 100/120V	1



## Appendix Biotage® Extrahera™ Settings

The methods described in this application note were automated on the Biotage<sup>®</sup> Extrahera<sup>™</sup>, using EVOLUTE<sup>®</sup> EXPRESS ABN 10 mg plates. Method performance was comparable.

Method	Total extraction time for 96 samples
EVOLUTE <sup>®</sup> EXPRESS Load-Wash-Elute method	30 mins 19 secs
EVOLUTE <sup>®</sup> EXPRESS `full' method	38 mins 5 secs

This appendix contains the software settings required to configure Extrahera to run the method described in this application note.

An importable electronic copy of this method for Extrahera can be downloaded from www.biotage.com

Method Name:	Biotin Full 10 mg ABN
Sample Plate/Rack:	2 mL x 96 well 400 µL
Extraction Media:	EVOLUTE ABN EXPRESS 96 Well Plate

od name otin Full 10r	ng ABN		Sample plat 2mL x 9	e/rack 6 well 400µL	-	Extraction media	BN EXPRESS 👻
eatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
Dn	Sample ty		Meth	od comment			
litioning On	Biotin		<b>.</b>				
libration		ample volume in plate/	rack (µL)				
Dn	220						
	Reuse san	NO					
Dn							
Dn							
n							
On							



#### Settings

0	
"Sample" Tab	
Sample Type:	Biotin
Starting Sample Volume (µL)	220
Reuse sample tips?	No
Method comment:	



## Screenshot

< Cancel	Ed	it SPE Meth	nod - Bioti	n Full 10mg	g ABN			Save >
Method name Biotin Full 10m	g ABN		Sample plate 2mL x 9	e/rack 6 well 400µL	-	Extraction media		ESS 👻
Pretreatment	Sample	Pretreatment	Conditioning		Load		Elution	Dispose solvent tip
Conditioning	Number o	f steps		step		step?	No	after each step?
quilibration On	Solvent 1% For	mic acid	<u>.</u>					
oad On	Volume (µL	)						
/ash On	Mix numbe	r of times Mix volume	(µL)					
lution	Wait time (	min)						
On								

### Settings

	Pre-treatment	Activated
	No. of steps	1
	Pause after last step	No
	Dispose tips after last step	No
_		
	Solvent	
1	1% Formic Acid	

1 190	FORTING ACIU				
2					
3					
4					
		1	2	3	4
Volume (µ	ıL)	220			
Mix numb	er of times	0			
Mix volum	ne (µL)	0			
Wait time	(min)	0			

d name			Sample plat	e/rack	-	Extraction media	
tin Full 10	mg ABN		2mL x 9	6 well 400µL	-	EVOLUTE A	BN EXPRESS 👻
atment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
n	Number of	steps Pressure (I	bar)				Dispose solv after each st
ioning	1	- 0.7					
n	1 Solvent		_				
oration	Methan	iol	-				
n 🗾	Volume (µL)	Collect in p	osition				
_	500	D (Was	👻				
n	Positive pre time (s)	ssure Advanced p settings	pressure				
	60	Edit	L				
n	Repeat (nur		this				
1	times)	step?	No				
n			NO				

	Conditioning		Activ	ated	
	No. of steps		1		
	Pressure (Bar)		0.7		
	Dispose tips af	ter each step	No		
	Solvent				
1	Methanol				
2					
3					
4					
		1	2	3	4
Volur	ne (µL)	500			
Colle	ct in position	D			

Pressure time (s)	60		
Repeat	1		
Pause after this step	No		

'Advanced Settings'



ethod name Biotin Full 10	mg ABN		Sample plat 2mL x 9	e/rack 6 well 400µL	-	Extraction media	BN EXPRESS 👻
etreatment	Sample I	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number of st	Pressure (b	ar)				Dispose solven after each step N
On	1 Solvent 1% Formi	ic acid	-				
On	Volume (µL)	Collect in pr	osition				
On <b>C</b>	500 Positive press time (s)	D (Was. ure Advanced p settings					
sh On	60	Edit					
tion	Repeat (numb times)	er of Pause after step?	No				
On							

Equilibration	Activated
No. of steps	1
Pressure (Bar)	0.7
Dispose tips after each step	No
Dispose tips after each step	No

Solvent				
1 1% Formic a	cid			
2				
3				
4				
	1	2	3	4
Volume (µL)	500			
Collect in position	D			
Pressure time (s)	60			
Repeat	1			
Pause after this ste	ep No			

#### 'Advanced Settings'

On Pause after each Positive pressure Advanced pressure	
Volume (µL) Pressure (bar) 00 400 0.7 Pause after each lead? Collect in position Pullibration	
On Pulse after each load? Collect in position Pestsure settings	
pullbration Positive reach Load 7 Collect in position Collect in position Collect in position Collect in collection Colle	
D (Mosta) 50 Edit	
Premix? Number of times Tip conditioning?	
On Yes 3 👻 No	
Ash Rinsing? Rinse volume (µL) Conditioning solvent	
On No 0 -	
lution Rinse solvent	
On T	

Load	Activated
Pressure (Bar)	0.7
Pause after each load	No
Volume (µL)	400
Collect in position	D
Positive pressure time (s)	60
Premix	Yes
Number of times	3
Rinsing	No
Rinse volume (µL)	N/A
Rinse solvent	N/A
Tip Conditioning	No
Conditioning solvent	N/A

'Advanced Settings'



Method name				mple plate/rack		_	Extraction media	1	-
Biotin Full 10	ng ABN		2	mL x 96 wel	l 400μL	*	EVOLUTE A	BN EXPRE	SS 👻
Pretreatment	Sample Pret	treatment	Conditi	oning Equ	ilibration	Load	Wash (2)	Elution	
On	Number of steps	Pressure (b	oar)	Plate dry after la wash?	st Plate dry	time (s)			ispose solvent ti Rer each step?
Conditioning	2 -	0.7		Yes	300			[	No
On	1 Solvent			2 Solvent					
quilibration	Water		-	Water-Me	OH (95:5)	<b>.</b>			
On	Volume (µL)	Collect in p	osition	Volume (µL)	Collect	in position			
oad	500	D (Was.		500	D (W	las 🝷			
On	Positive pressure time (s)	Advanced p settings	ressure	Positive pressu time (s)	re Advanci settings	ed pressure			
Vash	60	Edit		60	E	dit			
On	Repeat (number of times)	Pause after step?	this	Repeat (numbe	r of Pause a step?	fter this			
Iution	1 -	stepr	No	1	- Step:	No	r I		
On									

	Wash		Activa	ted	
	No. of steps		2		
	Pressure (Bar)		0.7		
	Plate dry after la	e dry after last wash			
	Plate dry time (s)	late dry time (s)			
	Dispose tips after	r each step	No		
_		_	_	_	_
	Solvent				
1	Water				
2	Water-MeOH (95	:5)			
3					
4					
		1	2	3	4
		1	2	3	4
Volur	me (µL)	500	500		
Colle	ct in position	D	D		
Press	sure time (s)	60	60		
Repe	at	1	1		
Paus	e after this step	No	No		

'Advanced Settings'

ethod name Biotin Full 10	mg ABN		Sample plat	e/rack 6 well 400µL	-	Extraction media	BN EXPRESS
retreatment	Sample Pre	treatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number of steps	Pressure (b	ar) Plate dry elution?		y timė (s)		Dispose solvent tip: after each step?
On	1 .	0.3		No 0			No
ullibration	Solvent 0.1% NH4OH	00/10 112					
On	Volume (µL)	Collect in po					
ad	200	В	÷				
On	Positive pressure time (s)	Advanced pr settings	essure				
on Direction	60	Edit.					
ition	Repeat (number of times)	F Pause after step?	this				
On	1 -		No				
-							

	Elution		Activated	i		
	No. of steps		1			
	Pressure (Bar)	Pressure (Bar)		Advanced		
	Plate dry after las	st elution	No			
	Plate dry time (s)		N/A			
	Dispose tips after	each step	No			
		_				
	Solvent					
1	Solvent					
2	0.1% NH4OH 90/	10 H20/MeOH				
3						
4						
		1	2	3	4	
Volur	ne (µL)	200				
Posit	ion	В				
Press	sure time (s)	Adv.				
Repe	at	1				
Pause	e after this step	No				
	<b>`Advanced Sett</b>	ings'				

0.3 bar for  $60\ s$  then  $1.0\ bar$  for  $40\ s$  then  $3.0\ bar$  for  $30\ s$ 



## Solvent Properties

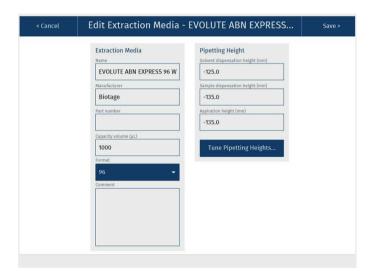
	Solvent Description	
1	1% Formic acid	
2	Methanol	
3	Water	
4	Water-MeOH (95:5)	
5	0.1% NH4OH 90/10 H20/MeOH	
6		TITI
7		
8		
9		
10		

Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type		Refill	able				N	on Refillab	le	
Capacity	N/A	N/A	N/A	N/A	N/A					
Aspiration flow rate (mL/min)	10	10	10	10	10					
Dispense flow rate (mL/min)	20	20	20	20	20					
Lower air gap flow rate (mL/min)	20	20	20	20	20					
Lower air gap volume (µL)	5	5	5	5	5					
Upper air gap flow rate (mL/min)	20	120	20	20	120					
Upper air gap volume (µL)	100	100	100	100	100					
Upper air gap dispense pause	300	300	300	300	300					
Conditioning?	Yes	Yes	Yes	Yes	Yes					
Conditioning number of times	2	3	2	2	3					
Conditioning flow rate (mL/min)	20	20	20	20	20					
Conditioning volume (%)	100	100	100	100	100					
Aspirate post dispense	Yes	Yes	Yes	Yes	Yes					
Chlorinated	No	No	No	No	No					
Serial dispense	No	No	No	No	No					



Sample	Air Gap	Aspirate	
Biotin	Lower air gap flow rate (mL/min)	Aspirate post dispense? Yes	
ample description	Lower air gap volume (µL)		
Biotin	5		
spiration flow rate (mL/min)	Upper air gap flow rate (mL/min)		
10	120		
Dispense flow rate (mL/min)	Upper air gap volume (µL)		
20	100		
	Upper air gap dispense pause (ms)		
	300		

"Sample Screen"	
Sample name	Biotin
Sample description	Biotin
Aspiration flow rate (mL/min)	10
Dispense flow rate (mL/min)	20
Lower air gap flow rate (mL/min)	20
Lower air gap volume (µL)	5
Upper air gap flow rate (mL/min)	120
Upper air gap volume (µL)	100
Upper air gap dispense pause	300
Aspirate post dispense	Yes



#### "Extraction Media Screen"

Name	EVOLUTE EXPRESS ABN 96 Well Plate
Manufacturer	Biotage
Part number	600-0010-PX01
Capacity volume (µL)	1000
Format	96
Comment	
Solvent dispensation height (mm)	-125.0
Sample dispensation height (mm)	-135.0
Aspiration height (mm)	-135.0

Sample Plate/Rack	Pipetting Height Aspiration height (mm)	
2mL x 96 well 400µL	-161.0	
Capacity volume (µL)	Pretreatment dispensation height (mm)	
1800	-135.0	
Format		
96 🗸	Tune Pipetting Heights	
	_	

"Sample Plate/Rack" Screen	
Name	2 mL x 96 well 400uL
Capacity volume (µL)	1800
Format	96
Aspiration height (mm)	-161.0
Pretreatment dispensation height (mm)	-135.0



< Cancel	Edit Pipette Tip - 1000 µL Biotage tip	Save >
	Pipette Tip Name 1000 µL Biotage tip	
	Manufacturer Biotage	
	Part number	
	Capacity (µL)  1000	
	Length (mm) 95	

"Pipette tip" Screen	
Name	1000 µL Biotage Tip
Manufacturer	Biotage
Part number	414141
Capacity (µL)	1000
Length (mm)	95

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