

Protein extraction

Thaw samples at 37° C. When there is still a frozen piece about the size of a pea, slowly add 7 mls of warm RPMI medium with L-glutamine but without serum and swirl, transferring cells to another tube. Spin cells down at 450g for 15 minutes in a refrigerated centrifuge. Wash twice with 1 ml of RPMI with L-glutamine but without serum. Resuspend in 1 ml of cold 10% trichloroacetic acid (TCA) and leave on ice for 1 hour. Spin down, aspirate off TCA, add 1 ml of 90% acetone, break up the pellet by pipetting up and down and smashing the pellet with the side of the tip, trying to avoid losing material by having it go up the tip or stick to the end, spin down in a microfuge for 1 minute and aspirate off the acetone. Repeat the acetone wash. Add DTT to denaturant (5 µl of 1M DTT added to 300 µl of 8M urea, 50mM NH₄HCO₃), making up enough for what you need. Add DTT-denaturant mix to the cell pellet pipetting up and down until it is dissolved; the volume of DTT-denaturant you need will depend on the size of the cell pellet with a large pellet requiring ~400 µl and a smaller pellet requiring ~100 µl. Vortex the cells in denaturant, give them a quick spin to get the liquid down, carefully stir and pipet up and down a bit with a p200 Pipetman until everything is in solution. If the cells are still present in a big chunk rather than having gone into solution, add more DTT-denaturant. Incubate the cells at 56° for 30 minutes. Make up a solution of 100 mM iodoacetamide in the dark, and add a volume of this iodoacetamide solution that is half of the volume of denaturation-DTT mix that was used. Incubate 30 minutes in the dark at room temperature. Mix 50mM NH₄HCO₃ with 1M CaCl₂ (1.4 µl of 1M CaCl₂ for each ml of 50mM NH₄HCO₃) and add 800 µl to each tube. Add 150 µl of denatured protein mix to each tube above and mix. Add 333 µl of 0.1% acetic acid to one container (100 µg) of Trypsin Gold (Promega), mix it up, and add 30 µl to the cells diluted down with NH₄HCO₃- CaCl₂, invert several times, and incubate in a 37° water bath overnight. Add 3 µl of glacial acetic acid to each tube, invert several times, and spin down in a microfuge for 10 minutes. Add 1 ml 100% acetonitrile + 0.1% formic acid, 0.1% trifluoroacetic acid to 3 ml SPEC C18 columns (Varian) with 15 mg sorbent (1 column per sample) and let it drip through completely. Add 1 ml 100% H₂O + 0.1% formic acid, 0.1% trifluoroacetic acid and let it drip through completely. Transfer the supernatant from the centrifuged peptide samples (there should be a bit of a pellet, which you do not want to transfer) to columns (one column per sample) and let it drip through. (This can take a long time - if it's not going through at all, one can apply a very light vacuum to get it started, but do not apply so much of a vacuum that the whole solution sucks through in a few seconds, because that won't give the peptides enough opportunity to bind to the column.) Fill the column with 100% H₂O + 0.1% formic acid, 0.1% trifluoroacetic acid and let it drip through. Elute with 1 ml of 50%

acetonitrile/50% H2O + 0.1% formic acid, 0.1% trifluoroacetic acid. Dry down in a speed vac and resuspend in 5% acetonitrile, 0.1% formic acid.

Mass spectrometry method

Creator: LTQ Orbitrap XL

MS Run Time (min): 105.00

Sequence override of method parameters not enabled.

Divert Valve: not used during run

Contact Closure: not used during run

Syringe Pump: not used during run

MS Detector Settings:

Real-time modifications to method disabled

Stepped collision energy not enabled

Additional Microscans:

MS2 0 0

MS3 0 0

MS4 0 0
MS5 0 0
MS6 0 0
MS7 0 0
MS8 0 0
MS9 0 0
MS10 0 0

Segment 1 Information

Duration (min): 105.00

Number of Scan Events: 7

Tune Method: 20070926_NSI tune_caffeine_3ul min

Scan Event Details:

1: FTMS + p norm !corona !pi res=100000 o(300.0-2000.0)

CV = 0.0V

2: ITMS + c norm !corona !pi Dep MS/MS Most intense ion from (1)

Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

3: ITMS + c norm !corona !pi Dep MS/MS 2nd most intense ion from (1)

Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

4: ITMS + c norm !corona !pi Dep MS/MS 3rd most intense ion from (1)

Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

5: ITMS + c norm !corona !pi Dep MS/MS 4th most intense ion from (1)

Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

6: ITMS + c norm !corona !pi Dep MS/MS 5th most intense ion from (1)

Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

7: ITMS + c norm !corona !pi Dep MS/MS 6th most intense ion from (1)

Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

Data Dependent Settings:

Use separate polarity settings disabled

Parent Mass List: (none)

Reject Mass List: (none)

Neutral Loss Mass List: (none)

Product Mass List: (none)

Neutral loss in top: 3

Product in top: 3

Most intense if no parent masses found not enabled

Add/subtract mass not enabled

FT master scan preview mode enabled

Charge state screening enabled

Monoisotopic precursor selection not enabled

Non-peptide monoisotopic recognition not enabled

Charge state rejection not enabled

Global Data Dependent Settings:

Use global parent and reject mass lists not enabled

Exclude parent mass from data dependent selection not enabled

Exclusion mass width by mass

Exclusion mass width low: 1.50

Exclusion mass width high: 1.50

Parent mass width by mass

Parent mass width low: 0.50

Parent mass width high: 0.50

Reject mass width relative to mass

Reject mass width relative to low (ppm): 10.0

Reject mass width relative to high (ppm): 10.0

Zoom/UltraZoom scan mass width by mass

Zoom/UltraZoom scan mass width low: 5.00

Zoom/UltraZoom scan mass width high: 5.00

Neutral Loss candidates processed by decreasing intensity

Neutral Loss mass width by mass

Neutral Loss mass width low: 0.50

Neutral Loss mass width high: 0.50

Product candidates processed by decreasing intensity

Product mass width by mass

Product mass width low: 0.50

Product mass width high: 0.50

MS mass range: 0.00-1000000.00

MSn mass range by mass

MSn mass range: 0.00-1000000.00

Analog UV data dep. not enabled

Dynamic exclusion enabled

Repeat Count: 1

Repeat Duration: 30.00

Exclusion List Size: 500

Exclusion Duration: 180.00

Exclusion mass width by mass

Exclusion mass width low: 1.50

Exclusion mass width high: 1.50

Expiration Count: 10

Expiration S/N Threshold: 2.0

Isotopic data dependence not enabled

Mass Tags data dependence not enabled

Custom Data Dependent Settings:

Not enabled _____

Method

RunTime: 105.00

AcquityBSMMethod

Scale: ScaleNano_1

AuxPump: true

HTM: false

NanoParameters

VariableFlowFactor: 0.10

TrappingMode: TrappingMode_2

EnablePeakParking: false

SampleLoadingTime: 10.00

BSMTrappingFlowRate: 2.500

BSMTrappingCompositionA: 100.0

BSMTrappingCompositionB: 0.0

TrappingLowPressureLimit: 0

TrappingHighPressureLimit: 10000

FlowRateADataEnable: false

FlowRateBDataEnable: false

TemperatureADataEnable: false

TemperatureBDataEnable: false

AuxPumpAParameters

AuxPumpRole: Auxiliary

AuxSolventName: Water

AuxSolventType: SolventType_2

AuxFlow: 0.000

AuxFlowSource: 1

AuxLowPressureLimit: 0

AuxHighPressureLimit: 10000

AuxPressureDataEnable: false

AuxFlowRateDataEnable: false

AuxPumpBParameters

AuxPumpRole: Lock Mass

AuxSolventName: Water

AuxSolventType: SolventType_2

AuxFlow: 0.000

AuxFlowSource: 1

AuxLowPressureLimit: 0
AuxHighPressureLimit: 10000
AuxPressureDataEnable: false
AuxFlowRateDataEnable: false
RunTime: 1.0
Comment
FlowSourceA: 1
FlowSourceB: 1
LowPressureLimit: 0
HighPressureLimit: 10000
SolventNameA: Water
SolventNameB: Acetonitrile
SolventTypeA: SolventType_2
SolventTypeB: SolventType_3
VentValve: VentValveSystem_2
DegasserEnable: true
InitialSwitch1: SwitchNoChange_4
InitialSwitch2: SwitchNoChange_4
InitialSwitch3: SwitchNoChange_4
SealWashPeriod: 5.0
Analog1: AnalogPressure_2
Analog2: AnalogB_4
SystemPressureDataEnable: false
FlowRateDataEnable: false
PercentADataEnable: false
PercentBDataEnable: false
LeftAPressureDataEnable: false
RightAPressureDataEnable: false

LeftBPressureDataEnable: false

RightBPressureDataEnable: false

DegasserPressureDataEnable: false

GradientTable

GradientRow

Time: Initial

Flow: 0.300

CompositionA: 100.0

CompositionB: 0.0

Curve

GradientRow

Time: 1.00

Flow: 0.300

CompositionA: 95.0

CompositionB: 5.0

Curve: 6

GradientRow

Time: 45.00

Flow: 0.300

CompositionA: 65.0

CompositionB: 35.0

Curve: 6

GradientRow

Time: 60.00

Flow: 0.300

CompositionA: 15.0

CompositionB: 85.0

Curve: 6

GradientRow

Time: 75.00

Flow: 0.300

CompositionA: 15.0

CompositionB: 85.0

Curve: 6

GradientRow

Time: 75.01

Flow: 0.300

CompositionA: 100.0

CompositionB: 0.0

Curve: 6

RunEvents: true

EventTable

AcquitySMMethod

RunTime: 1.0

Comment: Buffer A: 5% ACN, 0.1% FA in water<cr>Buffer B: 0.1% FA in acetonitrile

Mode: ModeSequential_0

LoopOption: LoopOptionPartial_1

LoopOffline: LoopOfflineDisable_0

WeakWashSolvent: Water

WeakWashVolume: 600

StrongWashSolvent: Acetonitrile

StrongWashVolume: 200

ColumnTemperature: HeaterOff_-1

ColumnTemperatureLimit: 5.0

SampleTemperature: 10.0

SampleTemperatureLimit: 5

Overfill: CustomDisable_-1

ColumnReequilibration: 1.0

DrawRate: DrawRateMedium_1

CustomRate: CustomDisable_-1

Illumination: IlluminationOff_0

NeedleDepth: CustomDisable_-1

PreAspirateAir: CustomDisable_-1

PostAspirateAir: CustomDisable_-1

ColumnTemperatureDataEnable: false

AmbientTemperatureDataEnable: false

SampleTemperatureDataEnable: false

ElevatorTemperatureDataEnable: false

SamplePressureDataEnable: false

Switch1: InitialSwitchState_5

Switch2: InitialSwitchState_5

Switch3: InitialSwitchState_5

Switch4: InitialSwitchState_5

Switch5: InitialSwitchState_5

Switch6: InitialSwitchState_5

HighPressureLimit: 5000

LowPressureLimit: 0

ChartOut: ChartOut_1

SampleTempAlarmEnable: false

ColumnTempAlarmEnable: false

RunEvents: true

EventTable

EventRow

Time: 1.01

Event: Event_2

Parameter1: Parameter1On_2

EventRow

Time: 1.02

Event: Event_2

Parameter1: Parameter1Off_1

Function: FunctionNone

ColumnManagerPresent: false

ColumnManager

ValvePosition: ValvePosition_1

SetColumnTemperature: HeaterOff_-1

EquilibrationTime: 0.1

EnableColumnTempDataChannel: false

EnableAlarmBand: true

SetAlarmBandDegC: 5.0

Switch1: InitialSwitchState_5

Switch2: InitialSwitchState_5

Switch3: InitialSwitchState_5

Switch4: InitialSwitchState_5

RunEvents: false

Last modified: 9/26/2007 by LTQ Orbitrap XL

MS Run Time (min): 105.00

Sequence override of method parameters not enabled.

Divert Valve: not used during run

Contact Closure: not used during run

Syringe Pump: not used during run

MS Detector Settings:

Real-time modifications to method disabled

Stepped collision energy not enabled

Additional Microscans:

MS2 0 0

MS3 0 0

MS4 0 0

MS5 0 0

MS6 0 0

MS7 0 0

MS8 0 0

MS9 0 0

MS10 0 0

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Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

3: ITMS + c norm !corona !pi Dep MS/MS 2nd most intense ion from (1)

Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

4: ITMS + c norm !corona !pi Dep MS/MS 3rd most intense ion from (1)

Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

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Activation Type: CID

Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

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Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

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Min. Signal Required: 500.0

Isolation Width: 2.00

Normalized Coll. Energy: 35.0

Default Charge State: 2

Activation Q: 0.250

Activation Time: 30.000

CV = 0.0V

Data Dependent Settings:

Use separate polarity settings disabled

Parent Mass List: (none)

Reject Mass List: (none)

Neutral Loss Mass List: (none)

Product Mass List: (none)

Neutral loss in top: 3

Product in top: 3

Most intense if no parent masses found not enabled

Add/subtract mass not enabled

FT master scan preview mode enabled

Charge state screening enabled

Monoisotopic precursor selection not enabled

Non-peptide monoisotopic recognition not enabled

Charge state rejection not enabled

Global Data Dependent Settings:

Use global parent and reject mass lists not enabled

Exclude parent mass from data dependent selection not enabled

Exclusion mass width by mass

Exclusion mass width low: 1.50

Exclusion mass width high: 1.50

Parent mass width by mass

Parent mass width low: 0.50

Parent mass width high: 0.50

Reject mass width relative to mass

Reject mass width relative to low (ppm): 10.0

Reject mass width relative to high (ppm): 10.0

Zoom/UltraZoom scan mass width by mass

Zoom/UltraZoom scan mass width low: 5.00

Zoom/UltraZoom scan mass width high: 5.00

Neutral Loss candidates processed by decreasing intensity

Neutral Loss mass width by mass

Neutral Loss mass width low: 0.50

Neutral Loss mass width high: 0.50

Product candidates processed by decreasing intensity

Product mass width by mass

Product mass width low: 0.50

Product mass width high: 0.50

MS mass range: 0.00-1000000.00

MSn mass range by mass

MSn mass range: 0.00-1000000.00

Analog UV data dep. not enabled

Dynamic exclusion enabled

Repeat Count: 1

Repeat Duration: 30.00

Exclusion List Size: 500

Exclusion Duration: 180.00

Exclusion mass width by mass

Exclusion mass width low: 1.50

Exclusion mass width high: 1.50

Expiration Count: 10

Expiration S/N Threshold: 2.0

Isotopic data dependence not enabled

Mass Tags data dependence not enabled

Custom Data Dependent Settings:

Not enabled

Method

RunTime: 105.00

AcquityBSMMethod

Scale: ScaleNano_1

AuxPump: true

HTM: false

NanoParameters

VariableFlowFactor: 0.10

TrappingMode: TrappingMode_2

EnablePeakParking: false

SampleLoadingTime: 10.00

BSMTrappingFlowRate: 2.500

BSMTrappingCompositionA: 100.0

BSMTrappingCompositionB: 0.0

TrappingLowPressureLimit: 0

TrappingHighPressureLimit: 10000

FlowRateADataEnable: false

FlowRateBDataEnable: false

TemperatureADataEnable: false

TemperatureBDataEnable: false

AuxPumpAParameters

AuxPumpRole: Auxiliary

AuxSolventName: Water

AuxSolventType: SolventType_2

AuxFlow: 0.000

AuxFlowSource: 1

AuxLowPressureLimit: 0

AuxHighPressureLimit: 10000

AuxPressureDataEnable: false

AuxFlowRateDataEnable: false

AuxPumpBParameters

AuxPumpRole: Lock Mass

AuxSolventName: Water

AuxSolventType: SolventType_2

AuxFlow: 0.000

AuxFlowSource: 1

AuxLowPressureLimit: 0

AuxHighPressureLimit: 10000

AuxPressureDataEnable: false

AuxFlowRateDataEnable: false

RunTime: 1.0

Comment

FlowSourceA: 1

FlowSourceB: 1

LowPressureLimit: 0

HighPressureLimit: 10000

SolventNameA: Water

SolventNameB: Acetonitrile

SolventTypeA: SolventType_2

SolventTypeB: SolventType_3

VentValve: VentValveSystem_2

DegasserEnable: true

InitialSwitch1: SwitchNoChange_4

InitialSwitch2: SwitchNoChange_4

InitialSwitch3: SwitchNoChange_4

SealWashPeriod: 5.0

Analog1: AnalogPressure_2

Analog2: AnalogB_4

SystemPressureDataEnable: false

FlowRateDataEnable: false

PercentADataEnable: false

PercentBDataEnable: false

LeftAPressureDataEnable: false

RightAPressureDataEnable: false

LeftBPressureDataEnable: false

RightBPressureDataEnable: false

DegasserPressureDataEnable: false

GradientTable

GradientRow

Time: Initial

Flow: 0.300

CompositionA: 100.0

CompositionB: 0.0

Curve

GradientRow

Time: 1.00

Flow: 0.300

CompositionA: 95.0

CompositionB: 5.0

Curve: 6

GradientRow

Time: 45.00

Flow: 0.300

CompositionA: 65.0

CompositionB: 35.0

Curve: 6

GradientRow

Time: 60.00

Flow: 0.300

CompositionA: 15.0

CompositionB: 85.0

Curve: 6

GradientRow

Time: 75.00

Flow: 0.300

CompositionA: 15.0

CompositionB: 85.0

Curve: 6

GradientRow

Time: 75.01

Flow: 0.300

CompositionA: 100.0

CompositionB: 0.0

Curve: 6

RunEvents: true

EventTable

AcquitySMMethod

RunTime: 1.0

Comment: Buffer A: 5% ACN, 0.1% FA in water<cr>Buffer B: 0.1% FA in acetonitrile

Mode: ModeSequential_0

LoopOption: LoopOptionPartial_1

LoopOffline: LoopOfflineDisable_0

WeakWashSolvent: Water

WeakWashVolume: 600

StrongWashSolvent: Acetonitrile

StrongWashVolume: 200

ColumnTemperature: HeaterOff_-1

ColumnTemperatureLimit: 5.0

SampleTemperature: 10.0

SampleTemperatureLimit: 5

Overfill: CustomDisable_-1

ColumnReequilibration: 1.0

DrawRate: DrawRateMedium_1

CustomRate: CustomDisable_-1

Illumination: IlluminationOff_0

NeedleDepth: CustomDisable_-1

PreAspirateAir: CustomDisable_-1

PostAspirateAir: CustomDisable_-1

ColumnTemperatureDataEnable: false

AmbientTemperatureDataEnable: false

SampleTemperatureDataEnable: false

ElevatorTemperatureDataEnable: false

SamplePressureDataEnable: false

Switch1: InitialSwitchState_5

Switch2: InitialSwitchState_5

Switch3: InitialSwitchState_5

Switch4: InitialSwitchState_5

Switch5: InitialSwitchState_5

Switch6: InitialSwitchState_5

HighPressureLimit: 5000

LowPressureLimit: 0

ChartOut: ChartOut_1

SampleTempAlarmEnable: false

ColumnTempAlarmEnable: false

RunEvents: true

EventTable

EventRow

Time: 1.01

Event: Event_2

Parameter1: Parameter1On_2

EventRow

Time: 1.02

Event: Event_2

Parameter1: Parameter1Off_1

Function: FunctionNone

ColumnManagerPresent: false

ColumnManager

ValvePosition: ValvePosition_1

SetColumnTemperature: HeaterOff_-1

EquilibrationTime: 0.1

EnableColumnTempDataChannel: false

EnableAlarmBand: true

SetAlarmBandDegC: 5.0

Switch1: InitialSwitchState_5

Switch2: InitialSwitchState_5

Switch3: InitialSwitchState_5

Switch4: InitialSwitchState_5

RunEvents: false