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Note

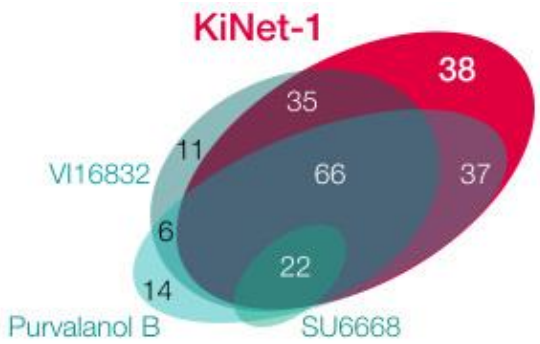
KiNet-1 must be first conjugated to a solid substrate such as NHS-activated Sepharose 4 Fast Flow (GE, Cat numbers: 17-0906-01, 17-0906-02). Please follow manufacture's instructions for conjugation protocols.

Disclaimer

The KiNet-1 reagent is an advanced experimental kit, designed for researchers that are skilled in isolating and identifying kinome signatures. Results between laboratories will vary. SYNkinase supports its research base, so please do not hesitate to contact us should you require more detail regarding KiNet-1 and its use.

Introduction

KiNet-1 lets you do more in less time and complexity than ever before. The human protein kinome is critical for the regulation of cell signaling and modulating a variety of key cellular processes. Perturbed kinase activity is strongly associated with human malignancies making protein kinases attractive targets for therapeutic intervention. KiNet-1 allows for rapid interrogation of cell signalling pathways and the identification of novel therapeutic strategies.



The unique pan-kinase affinity of KiNet-1 allows it to bind over 200 protein kinases within your samples. That's quite a few more than purvalanol B, VI16832 and SU6668 combined! KiNet-1 streamlines the kinome profiling process while greatly increasing the coverage of the expressed kinome.

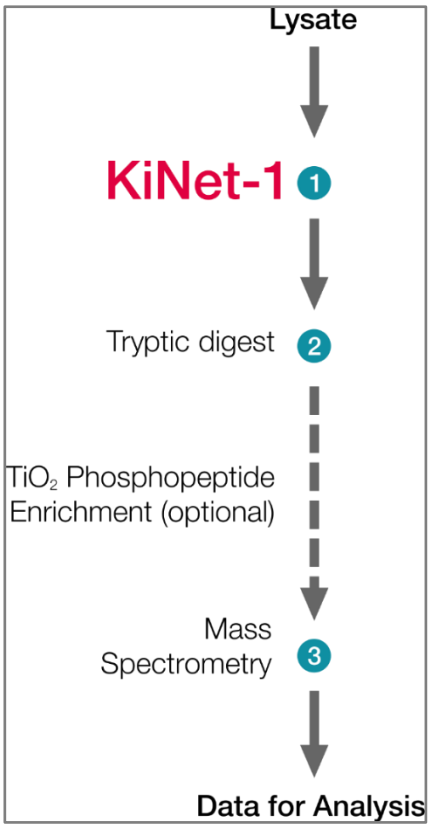
The KiNet-1 workflow starts by running sample lysate though a column of KiNet-1 beads. Next, enzymatic digestion of the eluent can be carried out in gel or solution. Following peptide extraction, samples can be analysed by mass spectrometry directly or following TiO2 enrichment for phosphopeptides. The kit is also wholly compatible with Western blot and ELISA methods of detection.

Small Scale Kinase Enrichment Protocol for Mass Spectrometry Discovery

The protocol described below is essentially as described in: *Zhang, L. et al. Journal of Proteome Research 12, 3104-3116, (2013)*. Although variances to this protocol will generally give the same experimental result, the protocol provided here is proven to return optimal kinase enrichment for downstream evaluation methods.

Buffer Recipes:

- **Lysis Buffer**: 50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, and 1 mM EGTA
- **Kinome Profiling Buffer**: lysis buffer supplemented freshly with 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM PMSF, 10 mM NaF, 50 ng/mL calyculin A, 1% phosphatase inhibitor mixture 2 (Sigma), and 2.5 mM Na_3VO_4
- **Washing Buffer A**: lysis buffer with 1 M NaCl plus 10 mM NaF and 0.1 mM Na_3VO_4
- **Washing Buffer B**: lysis buffer with 150 mM NaCl plus 10 mM NaF and 0.1 mM Na_3VO_4
- **Washing Buffer C**: 50 mM HEPES, 10 mM NaF and 0.1 mM Na_3VO_4
- **Elution Buffer**: 5 mM DTT, 0.5% SDS
- **SDS-PAGE Sample Buffer**: 60mM Tris-HCl (pH 6.8), 5% glycerol, 1.6% SDS, 100mM DTT.
- **Gel Washing Buffer**: 50 mM ammonium bicarbonate
- **Gel Destaining Buffer**: 50% of 200 mM ammonium bicarbonate and 50% of pure acetonitrile (v/v)
- **IAA Buffer**: 50 mM iodoacetamide (IAA) in 50 mM ammonium bicarbonate



Experimental Protocol:

1. Grow up cells in plates according to study's requirement. Remove growth media from plates, wash cells gently two times with ice-cold PBS. Remove as much PBS as possible.
2. Lyse cells using Kinome Profiling Buffer (see Buffer section). Centrifuge to remove debris (16,500g, 30 min, 4 °C) and filter the supernatant through a 0.22 µm PVDF membrane. Measure the concentration of proteins in lysate using Bradford assay.
3. Using 2 mg protein lysate, adjust the salt concentration to 1 M NaCl, ensuring a final protein concentration of between 1.5-2 mg/ml. Set aside on ice.

4. Spin 60 µl of 50% KiNet beads slurry (equivalent to 30 µl pure beads, 30 µl 80% EtOH) at 1000g for 1 min at room temperature, discard supernatant.

5. Wash once with 400 µl ddH2O followed by 400 µl of Washing Buffer A (see Buffer section).

6. Add cell lysate to the tube containing KiNet beads, incubate for 3 hrs in the dark at 4°C on a rotating wheel. Spin and discard supernatant.

7. Following incubation, wash the beads 3 times with 800 µl Washing Buffer A, 2 times with 800 µl Washing Buffer B, and once with 800 µl Washing Buffer C. Spin and discard washing solution each time.

8. Elute the resin-bound proteins using Elution Buffer. The Elution Buffer was added 80 µl at a time for 10 rounds; each round consisted of incubation in a 60°C water bath for 1 min with supernatant then transferred to a fresh tube. The eluted samples were pooled together and kept at -80°C.

9. Lyophilize the protein by freeze dryer once samples are completely frozen.

10. Resolubilize lyophilized protein in SDS-PAGE Sample Buffer, heat for 5 min at 95°C.

11. Run sample on a 10% SDS-PAGE gel, stain (Coomassie) and de-stain gel.

12. Cut out 16 bands from the sample lane. Cut each band into very fine pieces (1-2 mm), transfer to clean tubes.

13. Wash gel pieces in 200 µl Gel Washing Buffer for 5 min with vortexing. Spin and discard solution.

14. De-stain gel pieces using 200 µl Gel Destaining Buffer for 15 min then discard solution and repeat until gel pieces are fully de-stained.

15. Add 200 µl, 10mM DTT in 50 mM ammonium bicarbonate and incubate at room temperature for 30 min. Spin and discard solution.

16. Add 200 µl, 100% acetonitrile and incubate, with regular vortexing, for 10 min to dehydrate gel pieces. Spin and discard solution.

17. Add 200 µl IAA Buffer and incubate for 30 min at room temperature in the dark. Spin and discard solution.

18. Wash twice with 50 mM ammonium bicarbonate for 5 min. Spin and discard solution.

19. Dry gel pieces using centrifugal evaporatoror 20 min.

20. Add 30 µl of Sequence Grade Trypsin (20 ng/µl) to dried gel pieces. Incubate gel pieces at 4°C for 20 min.

21. Remove excess trypsin. Incubate gel pieces at 37°C overnight.

22. After overnight incubation, incubate gel pieces in 50 µl of 5% formic acid at room temperature for 15 min.

23. Add 50 µl of 100% acetonitrile to sample and incubate at room temperature for 15 min on a shaker.

24. Add another 100 µl of 100% acetonitrile to sample and incubate at room temperature for 15 min on a shaker.

25. Transfer formic acid/acetonitrile (200 µl total) solution to clean tubes. Centrifugal evaporator until dry.

26. Resuspend dried peptides in 20 µl of 0.1% formic acid and 2% acetonitrile.

27. Vortex briefly, then sonicate for 10 min in a sonication bath.

28. Run mass spectrometry of sample.

Mass Spectrometry Settings

The following protocol has been determined to provide optimal results on the Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer. Other instruments, or alternative downstream identification methods, are also known to work. For any further technical advice, please contact SYNkinase on info@synkinase.com. SYNkinase will update this datasheet as new protocols / methods are validated.

- **Overall Global Settings:**

Use Lock Masses	Off
Chrom. Peak Width (FWHM) Time	15s
Method Duration	65.00 min

- **Experimental Settings (FULL MS / DD-MS² (TOPN)):**

General	
Runtime	0 to 65 min
Polarity	Positive
In-source CID	0.0 eV
Default charge state	2
Inclusion	-
Exclusion	-
Tags	-
Full MS	
Microscans	1
Resolution	70,000

AGC Target	3e6
Maximum IT	120 ms
Number of Scan Ranges	1
Scan Range	375 to 1800 m/z
Spectrum Data Type	Profile
dd-MS² / dd-SIM	
Microscans	1
Resolution	17,500
AGC target	1e5
Maximum IT	120 ms
Loop Count	12
MSX Count	1
TopN	12
Isolation Window	2.5 m/z
Fixed First Mass	-
NCE	27.0
Stepped NCE	-
Spectrum Data Type	Profile
dd Settings	
Underfill Ratio	1.0%
Intensity Threshold	8.3e3
Apex Trigger	-
Charge Exclusion	unassigned, 1, 6 - 8, >8
Peptide Match	preferred
Exclude isotopes	on
Dynamic exclusion	15.0s
If Idle	Do not pick others

- **Setup:**

Tunefiles (General)	
Switch Count	0
Base Tunefile	[Path]
Contact Closure (General)	
Used	False
Start in Closed	True
Switch Count	0
Syringe (General)	
Used	False
Start in Off	True

Stop at End of Run	False
Switch Count	0
Syringe (Pump Setup)	
Syringe Type	Hamilton
Flow Rate	3.000 uL/min
Inner Diameter	2.303 mm
Volume	250 uL
Divert Valve A (General)	
Used	False
Start in 1-2	True
Switch Count	0
Divert Valve B (General)	
Used	False
Start in 1-2	True
Switch Count	0
Lock Masses	
371.10124 m/z	Start 0 min – End 120.00 min
445.12003 m/z	Start 0 min – End 120.00 min
536.16537 m/z	Start 0 min – End 120.00 min