First Edition, Version 1.04 November 2017

Sample Preparation Kit Pro

For High-Throughput Mass Spectrometry Proteomics

MANUAL

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Sample Preparation Kit Pro Components

Sample Preparation Kit Pro	Part No: Ki-3013 Sufficient for analysis of 96 samples Allows processing of two batches (2x 48)
Alkylation Solution	2x 10 ml tube, yellow cap
Reduction Stock Solution	2x 0.5 ml tube, orange cap
LC Solution	4x 10 ml tube, clear cap
10x Dilution Buffer	2x 10 ml tube, green cap
Denature Buffer	2x 10 ml tube, violet cap
96-well MACROSpin Plate	1x with 2x 96-well plate included
96-well Plate	3x 96-well plate packaged separately
96-well Plate Sealer	6x plate sealer
Manual	Available at www.biognosys.com/shop/sample-preparation-kit-pro

Storage and Quality Control of Sample Preparation Kit Pro

Immediately after receiving the kit store:

- Reduction Stock Solution and Alkylation Solution at **+4°C** and **protected from light**
- All other components should be stored dry at room temperature (15–25°C)

In accordance with Biognosys' Quality Management System, each lot of the kit is tested against predetermined specifications to ensure consistent product quality.

Use Limitations

The Sample Preparation Kit Pro is intended for mass spectrometry proteomics applications and research use only. This product is not intended for the diagnosis, prevention, or treatment of a disease. All due care and attention should be exercised in the handling of the products.

Product Warranty and Satisfaction Guarantee

Biognosys guarantees the performance of the product when following the instructions and protocols described in this product manual. However, the user must determine the suitability of the product for the intended use. Should the product fail to perform satisfactorily due to any reason other than misuse, Biognosys will replace it free of charge. Biognosys reserves the right to change, alter, or modify any product to enhance its performance and design.

If you have questions about product specifications or performance, please contact us at support@biognosys.com. We also encourage you to contact us if you have any suggestions for improving product performance or for its use in new applications and techniques.

Technical Assistance

Our Technical Department is composed of experienced scientists with extensive practical and theoretical expertise in proteomic technologies and bioinformatics. If you have any questions or experience any difficulties with the Sample Preparation Kit Pro please do not hesitate to contact us at support@biognosys.com call +41 44 738 20 40 or visit www.biognosys.com/shop/sample-preparation-kit-pro.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the material safety data sheet (MSDS) available online in PDF format at www.biognosys.com/shop/sample-preparation-kit-pro.

The following risk and safety phrases apply to components of the Sample Preparation Kit Pro.

10X Dilution Buffer: Harmful if swallowed.

Alkylation Solution: Toxic if swallowed, may cause allergy or asthma symptoms or breathing difficulties if inhaled, may cause an allergic skin reaction, may cause long lasting harmful effects to aquatic life.

Dissolution Buffer: Highly flammable liquid and vapour, causes serious eye irritation.

Reduction Stock Solution: Harmful if swallowed, causes skin and eye irritation, may cause respiratory irritation.

Introduction: Sample Preparation Kit Pro

Mass spectrometry based proteomics has become a prominent tool in biology and medicine in recent years due to its ability to identify and to precisely quantify thousands of proteins from different samples.

However, variability in sample preparation between experiments and individual samples remain an enormous source of experimental bias, especially in high-throughput applications. Since the proteomes of natural samples are extremely complex with typically 100'000s of peptides present, spanning over several orders of magnitude, it is challenging to establish a reliable method for preparing protein samples for analysis with mass spectrometry.

Biognosys' Sample Preparation Kit Pro is a simple-to-use standardized kit for high-throughput mass spectrometry proteomics of mammalian cell cultures and plasma/serum samples. The protocols work well with all mass spectrometry based proteomics applications such as SRM/MRM/PRM, HRM/DIA/SWATH and shotgun MS/MS.

Important Notes Before Starting

Before starting with the sample preparation, read through the steps carefully and make sure all the required reagents and equipment are available.

Use only highest purity solvents and water (ideally LC-MS grade) throughout the protocol to prepare buffers and solutions.

The kit components are sufficient for 96 human plasma or cell culture sample preparations.

The kit is designed to be used in 2 batches of 48 samples each. All reagents are supplied in two or four tubes / vials. All plates should be sealed after the first use and stored appropriately until the second usage. Note that when starting processing the second batch wells of the 96-well plate matching the unused part of the MACROSpin plate should be used.

To process 96 samples as a single batch all provided reagents are needed.

Additionally Required Laboratory Equipment and Consumables

Multichannel (5 µl – 200 µl) and single channel pipettes (0.5 µl – 1000 µl) with corresponding tips
Liquid trays for multichannel pipettes
Glass syringe (100 µl)
pH paper (recommended universal pH indicator paper pH 1-10 with colour scale) or pH meter with small combined glass electrode
Vortex mixer
15 ml plastic tubes
Benchtop centrifuge
Thermomixer at +37°C compatible with 96-well plates
Centrifuge with plate rotor and cooling option (+4°C)
Vacuum centrifuge with plate rotor
LC-MS vials or autosampler compatible with 96-well plate

Additionally Required Reagents, Solvents and Solutions

Sequencing grade modified trypsin, stock solution at 0.4 µg/µl (recommended Promega Product Catalog#V5113)
10% (v/v) TFA solution, prepare at least 2 ml for 48 samples (Note 1, Note 2)
Methanol, at least 15 ml for 48 samples
Water
Acetonitrile
C18 Cleaning Solution, prepare at least 15 ml for 48 samples (Note 1) ♦ 80% (v/v) Acetonitrile ♦ 0.1% (v/v) TFA (Note 2, Note 3) ♦ In water

Note 1
Solution can be stored at room temperature for up to one year.

Note 2
Use a glass syringe to pipette strong acidic solutions like concentrated TFA.

Note 3
Use 10% (v/v) TFA solution.

C18 Washing Solution, prepare at least 60 ml for 48 samples (Note 1)

- 1% (v/v) Acetonitrile
- 0.1% (v/v) TFA (Note 2, Note 3)
- In water

C18 Elution Solution, prepare at least 20 ml for 48 samples (Note 1)

- 50% (v/v) Acetonitrile
- 0.1% (v/v) TFA (Note 2, Note 3)
- In water

Plasma Sample Preparation Procedure

Sample Requirements

Plasma or serum, 10 µl per sample.

A. Denaturation, reduction and alkylation

1. Dissolve 10x Dilution Buffer with water to a total volume of 5 ml (Note 4), vortex until solubilized.
2. Dissolve Reduction Stock Solution with 250 µl water, vortex, briefly spin down.
3. Prepare Denature Buffer:
   3.1. Add 500 µl of 10x Dilution Buffer from step 1 to the Denature Buffer tube. Keep 10x Dilution Buffer in fridge until further usage in step 14.
   3.2. Add 25 µl of Reduction Stock Solution to the Denature Buffer tube.
   3.3. Fill up Denature Buffer with 2.3 ml of water (Note 4, Note 5).
4. Using a multichannel pipette, add 90 µl/well of Denature Buffer to a **new** 96-well plate (Note 6).
5. Add 10 µl of a plasma sample in each well (Note 7, Note 8).
6. Gently shake the plate or vials on thermomixer for 1 min at room temperature.
7. Briefly spin down to collect all liquid at the bottom of the wells.
8. Incubate the plate at +37°C, 600 rpm on thermomixer for 30 min.
9. Let the samples cool to room temperature while preparing the Alkylation Solution:
   9.1. Fill up Alkylation Solution with water to 3 ml (Note 4), vortex, briefly spin down (Note 9).
10. Using a multichannel pipette, add 16 µl/well of Alkylation Solution.
11. Gently shake the plate on thermomixer for 1 min at room temperature.
12. Briefly spin down to collect all liquid at the bottom of the wells.
13. Incubate the plate at room temperature **in the dark** for 30 min.

B. Dilution

14. Prepare 1x Dilution Buffer by mixing in a separate tube 1 ml of 10x Dilution Buffer and 9 ml of water.
15. Using a multichannel pipette, add 150 µl/well of 1x Dilution Buffer to a **new** 96-well plate (Note 6).

Note 4
To solubilize the provided solid components, dissolve first in a small amount of the stated solvent and fill-up to final volume carefully.

Note 5
Warm up the tube to help solubilizing the reagent by holding the tube in warm (<40°C) tap water.

Note 6
If only 48 samples are processed at a time, seal the unused wells.

Note 7
Thawed plasma samples should not be left without Denature Buffer at room temperature for longer than 5 minutes.

Note 8
Randomize sample location on plate.

Note 9
Light sensitive, prepare shortly before usage and keep in dark.

16. Using a multichannel pipette, add 15 µl/well of denatured plasma sample (from Section A, step 13).

Optional: you might freeze remaining sample in the plate for a repeated analysis.

17. Check pH to be 8–9 in a few wells by pipetting 0.5 µl onto a pH paper or by using a pH-electrode (Note 10).
    17.1. If pH is below 8, adjust it in **all** wells using 5 µl of 10x Dilution Buffer.
    17.2. Check again in a few wells, repeat steps 17.1 and 17.2 if necessary.

Note 10
Avoid sample cross-contamination by using fresh tips or cleaning the electrode with water for every sample.

C. Digestion using endoprotease trypsin

18. Thaw trypsin (0.4 µg/µl) and spin down briefly.
19. Add 5 µl/well of trypsin to the 96-well plate from Section B using a single channel pipette.
20. Gently shake the plate on thermomixer for 1 min at room temperature.
21. Briefly spin down to collect all liquid at the bottom of the wells.
22. Incubate the plate at +37°C, 600 rpm in the thermomixer for 3 hours.
23. Acidify samples by adding 20 µl/well of 10% (v/v) TFA solution using a multichannel pipette (Note 11).
24. Check pH to be below 2 in a few wells by pipetting 0.5 µl onto a pH paper or by using a pH-electrode.
    24.1. If pH is above 2, adjust it in **all** wells adding 5 µl of 10% (v/v) TFA solution.
    24.2. Check again in a few wells, repeat steps 24.1 and 24.2 if necessary.
25. Gently shake the plate on thermomixer for 1 min at room temperature.
26. Briefly spin down to collect all liquid at the bottom of the wells.

Note 11
Foaming is possible; add 10% (v/v) TFA solution slowly.

Optional: if convenient store samples at -20°C until C18 clean-up.

D. Sample clean-up using 96-well MACROSpin plates

Spin plate preparation

27. Place 96-well MACROSpin Plate on top of a **new** 96-well plate.
28. Remove the aluminium protection from the wells that will be used.
29. Add 200 µl/well of methanol to each well, centrifuge at 100 x *g* for 1 min, and discard flow-through.

30. Using a multichannel pipette, add 200 µl/well of C18 Cleaning Solution, centrifuge at 100 x *g* for 1 min, and discard flow-through.
31. Using a multichannel pipette, add 200 µl/well of C18 Washing Solution, centrifuge at 400 x *g* for 1 min, and discard flow-through (Note 12).
32. Repeat step 31 two more times.

Sample loading to spin plate

33. Centrifuge (thawed) 96-well plate from Section C with your samples at 1000 x *g* for 1 min.
34. Place 96-well MACROSpin Plate on a **new** 96-well plate, keep the old plate for later steps (36 to 39).
35. Load samples (supernatant) on 96-well MACROSpin Plate using a multichannel 100 µl pipette, centrifuge at 400 x *g* for 1 min, **do not discard flow-through** (Note 12).
36. Place spin plate on the 96-well plate previously used during the spin plate preparation (steps 27 to 32).
37. Load flow-through from step 35 on 96-well MACROSpin Plate using a multichannel pipette, centrifuge at 400 x *g* for 1 min and then discard the flow-through (Note 12).
38. Add 200 µl/well of C18 Washing Solution using a multichannel pipette, centrifuge at 400 x *g* for 1 min and discard the flow-through (Note 12).
39. Repeat step 38 two more times.
    If applicable, seal the unused wells after step completion and store the plate upon further usage (Note 6).

Elution and sample preparation

40. Place 96-well MACROSpin Plate on a **new** 96-well plate.
41. Add 170 µl/well of C18 Elution Buffer using a multichannel pipette, centrifuge at 400 x *g* for 1 min, keep flow-through in 96-well plate.
42. Repeat step 41 once again, collecting all eluates in the same collection plate (Note 6).

Optional: if necessary store 96-well plate with samples at -20°C until drying.

43. Dry down the combined eluates using a vacuum centrifuge (Note 13, Note 14), each sample contains approximately 100µg of peptides.
44. Dissolve dried samples (step 43) in LC Solution (volume depends on application and LC-MS settings) by pipetting up and down with a multichannel pipette.
45. Gently shake the plate on thermomixer for 1 min at room temperature.

Note 12
You may increase the centrifugation force up to 1'000 x g in case of low flow rates through the MACROSpin plate.

Note 13
Transfer eluates to Eppendorf tubes, if necessary.

Note 14
Dried samples can be stored at -20°C until usage.

46. Centrifuge the dissolved samples at 4°C and 1000 x *g* for 20 min.
47. Transfer sample supernatants to LC-MS vials or to an autosampler-compatible 96-well plate. You can also store the samples at -20°C.

Mammalian Cell Culture Sample Preparation Procedure

Sample Requirements

Cell culture pellets, with **at least 100 µg** of total protein per sample (approximately 100'000 to 1'000'000 cells depending on the cell line). If possible do not use trypsin for collection of adherent cells as cell surface proteins are damaged (use cell scraper instead).

A. Denaturation

1. Dissolve 10x Dilution Buffer with water to a total volume of 5 ml (Note 15), vortex until solubilized.
2. Prepare Denature Buffer:
   2.1. Add 500 µl of 10x Dilution Buffer from step 1 to the Denature Buffer tube. Keep 10x Dilution Buffer in fridge until further usage in step 14.
   2.2. Fill up Denature Buffer with 2.3 ml of water (Note 15, Note 16).
3. Add 80 µl of Denature Buffer to each sample and vortex until cell pellet is re-suspended (approximately 1 min).
4. Leave the samples on room temperature for 30 min.

Optional: Transfer one drop of sample on a glass slide and check lysis with a microscope. If intact cells are still visible, additional lysis measures, like bead milling or ultra-sonication might be necessary

5. During incubation you can measure the total protein concentration using a total protein assay (like BCA or Bradford) according to the manufacturer's protocol.
6. Prepare 1x Dilution Buffer by mixing in a separate 15 ml tube 1 ml of 10x Dilution Buffer and 9 ml of water.
7. Dilute the samples with 1x Dilution Buffer to obtain 100 µl per sample with protein concentration of 1 µg/ µl and transfer diluted samples into a **new** 96-well plate (Note 17). Keep 1x Dilution Buffer in fridge until further usage.

Optional: If convenient you can freeze your samples on -20°C. After thawing vortex the samples for 2 min before further use.

B. Reduction and alkylation

8. Dissolve Reduction Stock Solution with 250 µl water, vortex, briefly spin down.
9. Add 0.5 µl of Reduction Stock Solution to each well.
10. Incubate the plate at +37°C, 600 rpm on thermomixer for 30 min.

Note 15
To solubilize the provided solid components, dissolve first in a small amount of the stated solvent and fill-up to final volume carefully.

Note 16
Warm up the tube to help solubilizing the reagent by holding the tube in warm (<40°C) tap water.

Note 17
If only 48 samples are processed seal the unused wells.

11. Let the samples cool to room temperature while preparing the Alkylation Solution:
    11.1. Fill up Alkylation Solution with water to 3 ml (Note 15), vortex, briefly spin down (Note 18).
12. Using a multichannel pipette, add 16 µl/well of Alkylation Solution.
13. Gently shake the plate on thermomixer for 1 min at room temperature.
14. Briefly spin down to collect all liquid at the bottom of the wells.
15. Incubate the plate at room temperature **in the dark** for 30 min.
16. Using a multichannel pipette, add 25 µl/well of 1x Dilution Buffer to a **new** 96-well plate (Note 17).
17. Using a multichannel pipette, add 25 µl/well of cell lysate sample (from step 15).

Optional: you might freeze remaining sample in the plate for a repeated analysis.

18. Check pH to be 8–9 in a few wells by pipetting 0.5 µl onto a pH paper or by using a pH-electrode (Note 19).
    18.1. If pH is below 8, adjust it in **all** wells using 5 µl of 10x Dilution Buffer.
    18.2. Check again in a few wells, repeat steps 17.1 and 17.2 if necessary.

Note 18
Light sensitive, prepare shortly before usage and keep in dark at a time,

Note 19
Avoid sample cross-contamination by using fresh tips or cleaning the electrode with water for every sample.

C. Digestion using endoprotease trypsin

19. Thaw trypsin (0.4 µg/µl) and spin down briefly.
20. Add 1 µl/well of trypsin to the 96-well plate from Section B using a single channel pipette.
21. Gently shake the plate on thermomixer for 1 min at room temperature.
22. Briefly spin down to collect all liquid at the bottom of the wells.
23. Incubate the plate at +37°C, 600 rpm in the thermomixer **overnight**.

    NEXT DAY:

24. Acidify samples by adding 20 µl/well of 10% (v/v) TFA solution using a multichannel pipette (Note 20).
25. Check pH to be below 2 in a few wells by pipetting 0.5 µl onto a pH paper or by using a pH-electrode.
    25.1. If pH is above 2, adjust it in **all** wells adding 5 µl of 10% (v/v) TFA solution.
    25.2. Check again in a few wells, repeat steps 24.1 and 24.2 if necessary.

Note 20
Foaming is possible; add 10% (v/v) TFA solution slowly.

26. Gently shake the plate on thermomixer for 1 min at room temperature.
27. Briefly spin down to collect all liquid at the bottom of the wells.

Optional: if convenient store samples at -20°C until C18 clean-up.

D. Sample clean-up using 96-well MACROSpin plates

Spin plate preparation

28. Place 96-well MACROSpin Plate on top of a **new** 96-well plate.
29. Remove the aluminium protection from the wells that will be used.
30. Add 200 µl/well of methanol to each well, centrifuge at 100 x *g* for 1 min, and discard flow-through.
31. Using a multichannel pipette, add 200 µl/well of C18 Cleaning Solution, centrifuge at 100 x *g* for 1 min, and discard flow-through.
32. Using a multichannel pipette, add 200 µl/well of C18 Washing Solution, centrifuge at 400 x *g* for 1 min, and discard flow-through (Note 21).
33. Repeat step 31 two more times.

Sample loading to spin plate

34. Centrifuge (thawed) 96-well plate from Section C with your samples at 1000 x *g* for 1 min.
35. Place 96-well MACROSpin Plate on a **new** 96-well plate, keep the old plate for later steps (36 to 39).
36. Load samples (supernatant) from step 34 on 96-well MACROSpin Plate using a multichannel 100 µl pipette, centrifuge at 400 x *g* for 1 min, **do not discard flow-through** (Note 21).
37. Place spin plate on the 96-well plate previously used during the spin plate preparation (steps 27 to 32).
38. Load flow-through from step 35 on 96-well MACROSpin Plate using a multichannel pipette, centrifuge at 400 x *g* for 1 min and then discard the flow-through (Note 21).
39. Add 200 µl/well of C18 Washing Solution using a multichannel pipette, centrifuge at 400 x *g* for 1 min and discard the flow-through (Note 21).
40. Repeat step 39 two more times.
    If applicable, seal the unused wells after step completion and store the plate upon further usage (Note 17).

Elution and sample preparation

41. Place 96-well MACROSpin Plate on a **new** 96-well plate.

Note 21
You may increase the centrifugation force up to 1'000 x g in case of low flow rates through the MACROSpin plate.

Note 22
Transfer eluates to Eppendorf tubes, if necessary.

Note 23
Dried samples can be stored at -20°C until usage.

42. Add 170 µl/well of C18 Elution Buffer using a multichannel pipette, centrifuge at 400 x *g* for 1 min, keep flow-through in 96-well plate.
43. Repeat step 41 once again, collecting all eluates in the same collection plate (Note 17).

Optional: if necessary store 96-well plate with samples at -20°C until drying.

44. Dry down the combined eluates using a vacuum centrifuge (Note 22, Note 23), each sample contains approximately 20µg of peptides.
45. Dissolve dried samples (step 44) in LC Solution (volume depends on application and LC-MS settings) by pipetting up and down with a multichannel pipette.
46. Gently shake the plate on thermomixer for 1 min at room temperature.
47. Centrifuge the dissolved samples at 4°C and 1000 x *g* for 20 min.
48. Transfer sample supernatants to LC-MS vials or to an autosampler-compatible 96-well plate. You can also store the samples at -20°C.

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