Integrative extraction of primary metabolites and proteins from Deinococcus radiodurans

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Abstract

This protocol describes a procedure how primary metabolites and proteins can be extracted from *Deinococcus radiodurans* simultaneously. Polar metabolites are purified and derivatized for GC-MS (TOF) analysis. Proteins are tryptically digested in-gel and peptides are prepared for bottom-up proteomics by LC-MS (orbitrap).

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Protocol

Day 0

Step 1.

1. Grow *Deinococcus radiodurans* in suitable medium (like TGB or TGY) in sufficient amount for the experiment - TGB recipe: 1 % tryptone, 0.6 % beef extract, 0.2 % glucose

Day 1 - Separating proteins from metabolites

Step 2.

1. Centrifuge at 3000 g / 4 °C / 3 min (in Falcon tubes)

2. Discard supernatant and dissolve pellet in 1.8 mL cold PBS (transfer to homogenization tube)

3. Centrifuge at 1500 g / 4 °C / 3 min

4. Repeat step 2 and 3 two times

5. Discard washing solution and resuspend pellet in 1 mL cold, freshly prepared MCW (methanol 2.5 : chloroform 1 : water 0.5)

6. Homogenize in bead beater (e.g. Roche MagNA Lyser 4x 7000 rpm, 30 sec; chill between cycles)

7. Agitate for 10 sec, incubate 15 min on ice (shake once a while)

8. Centrifuge 21000 g / 4 °C / 5 min

9. Separate supernatant (contains metabolites) from pellet (contains proteins and nucleic acids)

Day 1 - Metabolite Extraction

Step 3.

1. Add 400 µL water to the supernatant (phase separation)

2. Agitate shortly and centrifuge at 14000 g / 2 min

3. Transfer upper (polar) phase into a new Eppendorf tube (lower, apolar phase can be stored if desired)

4. Dry samples in speed-vac by using the following gradient

200 mbar	30 min
120 mbar	30 min
80 mbar	30 min
50 mbar	30 min
20 mbar	30 min
0.001 mbar	3-4 h

5. Store overnight at -20 °C

Day 1 - Protein Extraction

Step 4.

1. Resuspend Pellet in Trizol

2. Homogenize one more time in the bead beater to dissolve pellet completely (Roche MagNA Lyser 1x 7000 rpm, 30 sec)

3. Incubate on a turning wheel at RT / 5 min

4. Add 200 µL Chloroform and shake vigorously for 15 sec

5. Incubate on a turning wheel at RT / 2-3 min

6. Centrifuge at 12000 rpm / 4 °C / 15 min

7. Remove aquaous phase (contains nucleic acids)

8. Add 400 µL water and incubate on turning wheel at RT / 2-3 min

9. Centrifuge at 12000 rpm / 4 °C / 15 min and remove aquaous phase

10. Precipitate the proteins by adding 5x 100 mM ammonium acetate in MeOH

11. Incubate overnight at -20 °C

Day 2 - Metabolites

Step 5.

1. Acclimate sample at room temperature when using frozen samples to avoid condensation

2. Redissolve sample in methoximation reagent (40mg Methoxyaminhydrochlorid (CH_3ONH_2*HCL) in 1ml Pyridine) for methoximation

3. Incubate 90 min. at 30°C using a thermo shaker

4. Add 80 µL MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) for silylation

5. Incubate for 30 min at 37 °C

6. Centrifuge at 14000 g / 2 min

7. Transfer supernatant in GC- microvials and close them with crimp caps

Metabolites ready for GC-MS measurement

Day 2 - Proteins

Step 6.

1. Centrifuge 5000 g / 15 min / 4 °C. Carefully discard the supernatant by pipetting it out.
2. Wash the pellet with 1 mL of acetone. Pipette up and down or use a soft ultrasound bath to disaggregate the pellet. Centrifuge 5000 g / 5 min / 4 °C. Discard the supernatant.
3. Repeat step 2 two times.
4. Air-dry the pellets until the acetone is completely eliminated.
5. Store at -20 °C overnight

Day 3 - Protein Quantification

Step 7.

1. Resuspend protein pellet in 8 M Urea, 4% (w/v) SDS. (*Recommend starting with the addition of a low volume of buffer, 20-30µL. If the buffer become very viscous (you can invert the tube and nothing is coming down), or there is some undissolved proteins more buffer can be added. A complete pellet solubilisation may take up to 3 h in a thermal shaker (35 °C and 750 rpm), being also some occasional pipetting needed.)*
2. Centrifuge at 21000 g / 5 min to remove insoluble particles.
3. Perform a BCA with a standard curve

BCA is compatible with SDS, Bradford not. But no reducing substances like DTT or β-mercaptoethanol should be in the sample!

- Prepare Standard Working Reagent (SWR): Mix 100 vol. of reagent A with 2 vol. of reagent B. The solution is stable at room temperature for 1 week.

BCA Reagent A: [sodium bicinchoninate (0.1 g), $Na_2CO_3 \cdot H_2O$ (2.0 g), sodium tartrate (dihydrate) (0.16 g), NaOH (0.4 g), $NaHCO_3$ (0.95 g), made up to 100 mL. If necessary, adjust the pH to 11.25 with

$NaHCO_3$ or NaOH]

BCA Reagent B: [$CuSO_4$ 5·H_2O (0.4 g) in 10 mL of water]

Reagents A and B are stable indefinitely at RT.

- Add BSA standards to an ELISA plate. 0, 1, 2, 3, 4, 6, 8, 10, 15 and 20 µg of BSA (in the same buffer as sample is dissolved). Three technical triplicates are advised.
- Add 2 µL of sample to each well.
- Add 200 µL of SWR to each well. Shake the plate gently.
- Close the plate with a cap or aluminium foil to minimize evaporation. Incubate the plate 20 min at 60 °C (in an oven) or 40 min at 45 °C in the plate reader. Read absorbance at 562 nm.

Day 3 - SDS-PAGE

Step 8.

1. Prepare SDS-PAGE Gels

- 10x Running Buffer (TGS): [10 g of SDS, 30.2 g Tris-Base, 144 g of glycine, add mQH_2O up to 1 L]
- Resolving gel: [12.5 % acrylamide:bisacrylamide, 0.1 % SDS, 0.375 M Tris-HCl pH 8.8, 0.2 % TEMED, 0.073 % APS.] Avoid bubbles during casting and quickly cover the acrylamide with 2-propanol. Let the gels polymerize for 1 hour.
- Stacking gel: [5 % acrylamide:bisacrylamide, 0.2 % SDS, 0.225 M Tris-HCl pH 6.8, 0.2 % TEMED, 0.1 % APS]. Before adding the APS and TEMED discard the 2-propanol layer covering the resolving gels and briefly rinse with water. Then pour the stacking solution containing APS and TEMED, and carefully place the comb avoiding bubbles.

	mini-gels (0.75mm) (for 2 gels)		mini-gels (0.75mm) (for 4 gels)	
	12.5% separating gel	5% stacking gel	12.5% separating gel	5% stacking gel
30% acrylamide	3.125 mL	0.5 mL	6.25 mL	1 mL
mQH_2O	2.355 mL	1.063 mL	4.71 mL	2.126 mL
10% SDS	75 µL	60 µL	150 µL	120 µL
1.5 M Tris-HCl pH 8.8	1.875 mL	-	3.75 mL	-
0.5 M Tris-HCl pH 6.8	-	1.35 mL	-	2.7 mL
TEMED	15 µL	6 µL	30 µL	12 µL
10% APS	55 µL	21 µL	110 µL	42 µL
Total	7.5 mL	3 mL	15 mL	6 mL

2. Take proper amount of protein (40-80 µg) add 2 µL of 5x Laemmli buffer and add mQH_2O up to 10 µL. (5x Laemmli buffer: [80% (v/v) Glycerol, 20% (w/v) SDS, 20% (v/v) ß-Mercaptoethanol, 0.01% (w/v) bromophenol

blue])

3. Load samples and leave blank well between samples to avoid contaminations.
4. Run samples through stacking gel constant 40 V, so until they reach stacking-resolving interphase.
5. Run gel constant 80 V until bromophenol blue reach approximately 1 cm into the stacking gel.
6. Stop electrophoresis.
7. Immediately transfer the gel for staining. Incubate 30 min in shaking. (Coomassie staining solution: 40% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie R-250, in distilled water. Dissolve the Coomassie

in MeOH and then add the other components.]) *Recovery of the tryptic peptides is dramatically reduced in over fixed gels*
8. Destain for 80 min replacing destain every 20 min. (Coomassie destaining solution: [40% (v/v) MeOH, 2% (v/v) acetic acid in mQH_2O])
9. Wash the gel in mQH_2O for 30 min
10. Cut lanes out of the gel (can be stored at -20 °C for long term, before freezing cover with mQH_2O)

Day 4 - Protein Digestion

Step 9.

1. Chop the gel pieces with a scalpel over a glass plate. The final size of acrylamide pieces should be around 1 mm^3. Glass plate should be carefully cleaned after each gel piece. Avoid taking gel without proteins.
2. Transfer the gel pieces to a 1.5 mL LoBind tube and keep them on mQH_2O until all samples are processed.
3. Remove water and add 1 mL of 25 mM AmBic, incubate 15 min at 37 °C in a thermal shaker. Discard supernatant.
4. Add 1 mL of 25 mM AmBic, 50% ACN, incubate 15 min at 37 °C in a thermal shaker. Discard supernatant.

Repeat (*The supernatant must be almost transparent or with a very light blue colour, if the blue colour is intense, repeat again. Band destaining and cleaning is crucial for a good LC-MS analysis. In these steps not*

only coomassie, but also SDS and other contaminants are removed. If there is still blue colour inside the gel pieces after three washing steps, continue with the protocol, it will not interfere with protein digestion.

This usually happens when the gel pieces are not small enough.)
5. Dehydrate gel pieces with 300 µL 100% ACN incubate 5 min at RT in a shaker. Discard supernatant. (*At this point gel pieces should be much reduced in volume, hard, and completely white, having a*

“plastic” look. If

they are still translucent or soft repeat this step (you can use the pipette tip to test it).)
6. Reduce disulphide bonds with 20 mM DTT in 100 mM AmBic (cover gel pieces) at 37 °C for 30 min.
7. Add 500 µL of 100% ACN to shrink gel pieces to remove DTT.
8. Alkylate reduced Cys residues by adding 55 mM IAA in 100 mM AmBic (cover gel pieces) at RT in DARK for 60min.
9. Remove all IAA solution
10. Wash with 500 µL 25 mM AmBic, incubate 15 min at 37 °C in a thermal shaker. Discard supernatant.
11. Wash with 25 mM AmBic, 50% ACN, incubate 15 min at 37 °C in a thermal shaker. Discard supernatant.
12. Dehydrate gel pieces with 300 µL 100% ACN incubate 5 min at RT in a shaker. Discard supernatant.
13. Dry out in a SpeedVac for 5 min.
14. Add 50 µL of Trypsin Solution to each tube and incubate 15 minutes at 37 °C. If needed, add more trypsin solution until the gel pieces are completely rehydrated. Wait another 15 minutes. Completely cover the gel

pieces with trypsin buffer.[Trypsin Solution: dilute Trypsin Sequencing Grade (Roche 11 418 475 001) to 10-12.5 ng/µL. Add 1 mL of trypsin buffer to the vial, incubate on ice for 10 min and then pipet up and down to

completely resuspend the pellet. Transfer to a 2 mL LoBind tube. Add 1 mL of trypsin buffer to the vial, washing all the walls. Then transfer to the previous tube. Trypsin can be stored at -20 °C for 1 month. It is

recommended to make aliquots to avoid melt/freezing cycles that can damage the enzyme. Aliquots should be stored in low binding tubes.];[*Trypsin Buffer*: 25 mM NH_4HCO_3 (AmBic), 10% (v/v) acetonitrile, 5 mM

$CaCl_2$. Calcium must be added in last place to avoid precipitation. Prepare fresh.]
15. Incubate 14-16 h at 37 °C. Incubation in an oven is recommended, if a thermal block is used, cover it with aluminium foil to maintain the temperature also in the caps of the tubes. No shaking!

Day 5 - Peptide Extraction

Step 10.

1. Add 150 µL of 50% ACN, 1% FA to each tube with gel pieces and incubate 5 min at RT. (After incubation sonicate 3 min in a low intensity ultrasound bath.)
2. Centrifuge (21000 g / 1 min) and transfer the supernatant to a new tube
3. Transfer the supernatant to the same tube
4. Add 100 µL of 90% ACN, 1% FA and incubate for 5 min at RT. Transfer the supernatant to the same tube again.
5. Dry down in SpeedVac (same gradient as for metabolites mentioned in step 3)
6. Keep at -20 °C or proceed with desalting (long term storage at -80 °C)

Day 6 - Desalting

Step 11.

1. Add 75 µL of PS to peptide pellets. Incubate for 10 min on ice. (Peptide Solubilisation Buffer (PS): [4% (v/v) ACN, 0.25% (v/v) FA])
2. Re-suspend the pellet by pipetting and sonicate 3 min in a low intensity ultrasound bath. Incubate 5 min at RT and then keep on ice.
3. Use C18 stage tips (or 96-well plates for peptide desalting). Independently of the chosen support the protocol remains unchanged (only volumes should be adjusted). All of the steps at RT.
4. Plate activation with 400 µL MeOH x2, allow flowing to waste by gravity for 3 min then aspirate. (TIPS: 100 µL MeOH x2)
5. Plate equilibration with 400 µL mQH_2O x4, allow to flow to waste by gravity for 3 min then aspirate. (TIPS: 100 µL mQH_2O x2)
6. Sample binding: Pipette solution in the center of the well all of the volume in the tubes, including all of the insoluble parts that may remain in the solution. Confirm visually that the membrane absorbs the peptides.

Allow to flow to waste by gravity for 10 min then aspirate. (TIPS: pipet slowly up and down)
7. Sample desalting: with 400 µL of mQH_2O to each well x5. Do not increase the vacuum too high. (TIPS: 100 µL mQH_2O x2)

8. Last aspiration to total dryness, 1 min.
9. Sample recovery: Replace the under well tray with a clean one. Elute peptides with 200 µL of MeOH x2. Allow to flow by gravity for >5 min then aspirate. (TIPS: 50 µL MeOH x2)
10. Use vacuum to elute everything.

11. Transfer the eluted peptides to LoBind tubes.

12. Dry down in speed-vac

Peptides ready for LC-MS measurement