

PROTOCOL: Automated Protein Digestion (DIG)

Purpose

Enzymatically convert proteins into peptides prior to phosphopeptide enrichment, with associated biochemical steps to prevent formation of disulfide bonds.

Preparation

1. Prepare DIGMIX01 (100 mM DTT)
2. Prepare DIGMIX02 (200 mM iodoacetamide)
3. Prepare DIGMIX03 (25% TFA)
4. Thaw trypsin {DIG-M05} and hold at 4°C.
5. Prepare LT-BRAVO
 - 5.1. On the LT-BRAVO Eva {DIG-A02} load the device file "96LT_Bravo.dev". This file is located at C:\\VWorks Workspace\\Device Files\\.
 - 5.2. In the "Devices" page, click on "Agilent Bravo" and then "96LT Bravo". Select "Initialize all devices".
 - 5.3. Open the protocol file "LysateDigestion (8M Urea) 180F_Tips.pro". This file is located at C:\\VWorks Workspace\\Protocol Files\\LT_Bravo\\CF\\P100\\.

Materials

- HPLC-grade water, JT Baker, Cat. No. 4218-03 {DIG-M01}
- Dithiothreitol (DTT), Sigma-Aldrich, Cat. No. 43819-5G {DIG-M02}
- Iodoacetamide (IAM), Sigma-Aldrich, Cat. No. I1149-25G {DIG-M03}
- 50 mM Tris solution pH 8.0, diluted from Invitrogen, Cat. No. 15568-025 {DIG-M04}
- Trypsin 0.5 µg/µl, Promega, Cat. No. V5113 {DIG-M05}
- Trifluoroacetic acid (TFA), Sigma-Aldrich, Cat. No. T6508-25ML {DIG-M06}
- Agilent 96LT-180uL Filter Tips, Agilent Technologies, Cat. No. 19477-042 {DIG-M07}
- 96-Well 2.2mL Deep Well plate, VWR, Cat. No. 82006-448 {DIG-M08}
- 96-Well skirted PCR plate, Bio-rad, Cat. No. MSP9601 {DIG-M09}
- 384-Well Deep Well Microplate, Greiner Bio-One, Cat. No. 781270 {DIG-M10}
- 12-Column High Profile Reagent Reservoir, Axygen, Cat. No. RES-MW12-HP {DIG-M11}
- Axygen -80°C Rated Foil Seal, Axygen, Cat. No. PCRAS200 {DIG-M12}
- pH Test Strips, JT Baker, Cat. No. 4393-01 {DIG-M13}
- Breath-EASIER seal, Diversified Biotech, Cat. No. BERM-2000 {DIG-M14}

Assets

- BioMicroLab Rack Thawing Station {A01}
- Agilent LT-BRAVO Automated Liquid Handling Platform with VWorks4 {A02}
- Eppendorf Microcentrifuge {A03}
- Eppendorf Plate Shaker {A04}
- Thermo Scientific Savant SC210A Concentrator {A05}

Reagent Mixes

ID	Name	Step	Composition	Volume/Well	Use
DIGMIX01	100 mM DTT	DIG	15.4 mg/mL DTT {DIG-M02} in HPLC grade water {DIG-M01}	50 μ L	Reduction reagent; keep on ice or at 4°C.
DIGMIX02	200 mM IAM	DIG	36.99 mg/mL IAM {DIG-M03} in HPLC grade water {DIG-M01}	50 μ L	Alkylation reagent; keep on ice or at 4°C and in dark.
DIGMIX03	25% TFA	DIG	25% TFA {DIG-M06} in HPLC grade water {DIG-M01}	150 μ L	Digestion inactivating reagent; CAUTION ACID

Mix Preps and Mini-worksheets:

DIGMIX01 - 100 mM DTT

1. Weigh out at least 92.4 mg DTT {DIG-M02} to make at least 6 mL
2. Calculate amount of HPLC-grade water {DIG-M01} to add in mL by dividing amount weighed out by 15.4
 - Amount weighed: _____ mg
 - Divide by: 15.4
 - Water to add _____ mL

DIGMIX02 - 200 mM IAM

1. Weigh out at least 222 mg iodoacetamide {DIG-M03} to make at least 6 mL
2. Calculate amount of water {DIG-M01} to add in mL by dividing amount weighed out by 36.99
 - Amount weighed: _____ mg
 - Divide by: 36.99
 - Water to add _____ mL

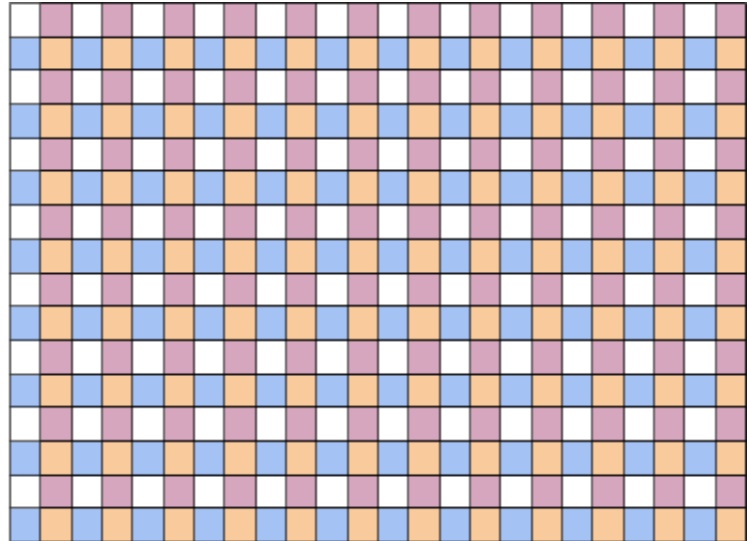
DIGMIX03 - 25% TFA

1. Pipette 13.5 mL of water {DIG-M01} into a conical vial.
2. Pipette 4.5 mL of TFA {DIG-M05} into the vial.

Procedure

1. Aliquot reagents into a 384-well plate {DIG-M10} according to the following layout for the entire plate:

(Q1) Blank	(Q2) 100mM Dithiothreitol (DTT) {Mix01} [50uL/well]
(Q3) 200mM Iodoacetamide (IAM) {Mix02} [50uL/well]	(Q4) 25% Trifluoroacetic Acid (TFA) {Mix03} [150uL/well]

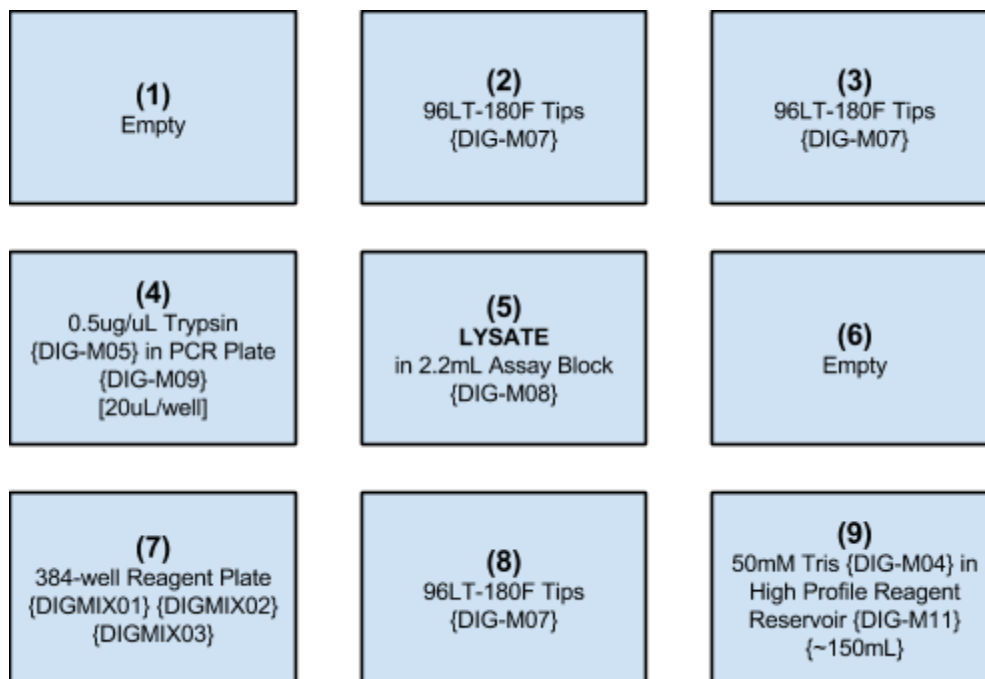


NB: Use a multi-channel pipette to deliver these reagents to the appropriate wells. Take care to deliver the correct reagent to the correct quadrant. Cover plate with foil and hold at 4°C if not using immediately.

2. Aliquot 20µL of trypsin {DIG-M05} to each well of a 96-well PCR plate {DIG-M09}.

NB: Keep trypsin on ice or at 4°C until it is added to samples.

3. Fill 12-column high profile reagent reservoir {DIG-M11} with ~150mL of 50mM Tris pH 8.0 solution {DIG-M04}
4. Assemble the deck of the LT-BRAVO according to the following layout:



5. On the LT-BRAVO, toggle to “Simulation is on” at the top of the screen from “Simulation is off”.
 - 5.1. Press Start and the Run Configuration Wizard will pop up. Press Finish.
 - 5.2. A pop up entitled “Set Initial Values for Variables” will appear. Set the number of “Columns” to the appropriate amount of sample columns and press ok.
 - 5.3. The simulation will run and provide feedback on any warnings or errors that the protocol may encounter. The only acceptable result is “No Errors”. If there are any, that come up, notify the key LT-BRAVO user and obtain help.
 - 5.4. Place black shroud over the instrument, to ensure darkness during reduction and alkylation steps.
6. On the LT-BRAVO, toggle back to “Simulation is off”.
7. Press GO. Follow prompts and enter number of columns being used on the plate. (DIG Automation Protocol Step 1).

NB: The protocol will pause after reduction (DIG Automation Protocol Step 2), alkylation (DIG Automation Protocol Steps 3, 4, 5), dilution (DIG Automation Protocol Step 6), and trypsin addition (DIG Automation Protocol Step 7) have occurred (~65 minutes).

8. After the protocol has paused, place a foil seal {DIG-M12} on the **LYSATE** plate and replace the plate in position 5 on the LT-BRAVO deck. (DIG Automation Protocol Step 7.6)
 - 8.1. The LT-BRAVO will move the plate to the heated shaker where it will incubate at 37°C for 15 hours (overnight). (DIG Automation Protocol Step 7.7).
9. On the next day, remove the **LYSATE** plate from the LT-BRAVO, vortex, and spin down.

NB: Replace tips at position 8 with a clean box of 180uL filter tips.

10. Remove the foil cover and replace the plate in position 6 at the heated shaker position.
 - 10.1. Press GO to continue the protocol.
 - 10.2. The LT-BRAVO will add 25% TFA to the **LYSATE** plate and quench the digestion reaction. (DIG Automation Protocol Step 8).
11. Remove the **LYSATE** plate from the LT-BRAVO, cover with foil {DIG-M12}, vortex thoroughly, and centrifuge for 1 to 2 minutes to ensure that all samples are acidified.
12. Remove foil carefully from the plate.
13. Test the pH of 3 random samples (aiming for pH less than 4).
 - 13.1. Use 0.2µL of sample to test pH using pH test strips {DIG-M13}.
 - 13.2. If sample pH is not acidic, vortex and centrifuge again until acidification is achieved
14. Keep **LYSATE** plate on ice or at 4°C until continuing to **PP96D**.

DIG Automation Steps LT-Bravo:

1. Define Variables
 - 1.1. Columns = 12
 - 1.2. ReagentVol = 25
2. Reduction
 - 2.1. Set head mode to all barrels
 - 2.1.1. `task.Headmode="1,2,8,"+Columns;`
 - 2.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Columns" to the appropriate number.
 - 2.2. Tips on from Position 2.
 - 2.3. Aspirate 25uL from quadrant 2 of position 7. (DTT)
 - 2.3.1. Volume = ReagentVol
 - 2.3.2. Post-aspirate volume = 5
 - 2.3.3. Distance from well bottom = 0.5
 - 2.3.4. Well selection = 1 selection: quadrant 2
 - 2.4. Dispense contents of tips to position 5. (LYSATE)
 - 2.4.1. Distance from well bottom = 2
 - 2.5. Tips off in Position 2.
 - 2.6. Shake plate from Position 5 at Position 6.
 - 2.6.1. 37degC, 800rpm, 30 minutes
3. Place plate at Position 5.
4. Alkylation
 - 4.1. Set head mode to all barrels
 - 4.1.1. `task.Headmode="1,2,8,"+Columns;`
 - 4.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Columns" to the appropriate number.
 - 4.2. Tips on from Position 2.
 - 4.3. Aspirate 25uL from quadrant 3 of position 7. (IAM)
 - 4.3.1. Volume = ReagentVol
 - 4.3.2. Post-aspirate volume = 5
 - 4.3.3. Distance from well bottom = 0.5
 - 4.3.4. Well selection = 1 selection: quadrant 3
 - 4.4. Dispense contents of tips to position 5. (LYSATE)
 - 4.4.1. Distance from well bottom = 2
 - 4.5. Mix 180uL at Position 5.
 - 4.5.1. Mix cycles = 10
 - 4.5.2. Distance from well bottom = 2
 - 4.6. Tips off in Position 2.
5. Incubate at Position 5 for 30 minutes in the dark.
6. Dilution
 - 6.1. Set head mode to all barrels
 - 6.1.1. `task.Headmode="1,2,8,"+Columns;`
 - 6.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Columns" to the appropriate number.
 - 6.2. Tips on from Position 3.

- 6.3. Loop 7 times changing tips every 7 times.
- 6.4. Aspirate 165uL from Position 9. (50mM Tris)
 - 6.4.1. Post-aspirate volume = 10
 - 6.4.2. Distance from well bottom = 1
- 6.5. Dispense contents of tips to Position 5. (LYSATE)
 - 6.5.1. Distance from well bottom = 2.
- 6.6. Loop End.
- 6.7. Tips off at Position 3.
7. Trypsin Digest
 - 7.1. Set head mode to all barrels
 - 7.1.1. `task.Headmode="1,2,8,"+Columns;`
 - 7.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Columns" to the appropriate number.
 - 7.2. Tips on from Position 8.
 - 7.3. Aspirate 10uL from Position 4. (Trypsin)
 - 7.3.1. Distance from well bottom = 1
 - 7.4. Dispense contents of tips to Position 5. (LYSATE)
 - 7.4.1. Distance from well bottom = 2
 - 7.5. Tips off at Position 8.
 - 7.6. Wait for user to press the "Go" button.
 - 7.6.1. "Cover digest plate in foil, then press GO."
 - 7.7. Shake plate from Position 5 at Position 4.
 - 7.7.1. 37degC, 800rpm, 15 hours.
8. Stop Digest
 - 8.1. Set head mode to all barrels
 - 8.1.1. `task.Headmode="1,2,8,"+Columns;`
 - 8.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Columns" to the appropriate number.
 - 8.2. Wait for user to press the "Go" button.
 - 8.2.1. "Spin down plate, remove foil cover, then press GO."
 - 8.3. Tips on from Position 8.
 - 8.4. Aspirate 75uL from quadrant 4 of Position 7. (25% TFA)
 - 8.4.1. Post-aspirate volume = 5
 - 8.4.2. Distance from well bottom = 0.5
 - 8.4.3. Well selection = 1 selection: quadrant 4
 - 8.5. Dispense contents of tips to Position 5. (LYSATE)
 - 8.5.1. Distance from well bottom = 2
 - 8.6. Mix 160uL at Positon 5.
 - 8.6.1. Mix cycles = 5
 - 8.6.2. Distance from well bottom = 1
 - 8.7. Tips off at Position 8.