H2020-MSCA-ITN-2020

Allostery in Drug Discovery

Above-Filter Digestion Proteomics

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Abstract: Above-Filter Digestion Proteomics (AFDIP) is a chemical proteomics method designed to identify drug targets and characterize drug-protein binding sites through differential trypsin digestion kinetics. This technique monitors how ligand binding affects protein accessibility to trypsin, with digestion rates typically decreasing at binding sites while potentially increasing at other locations due to allosteric structural changes.

Key applications:

- Drug target identification and validation
- Binding site localization (≤10 Å resolution, improving to ≤5 Å for larger proteins)
- Dose-response analysis for drug-target interactions
- Protein-metabolite interaction mapping
- Complementary approach to existing chemical proteomics methods (TPP, PISA, LiP)

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AFDIP Protocol: Above-Filter Digestion Proteomics for Drug Target Identification

1. **Cell Culture**
    1. Grow HeLa cells at 37°C in 5% CO_2
    2. Use DMEM supplemented with 2 mM L-Glutamine, 100 U/mL Pen/Strep, and 10% FBS
    3. Harvest cells when 80-90% confluent
2. **Proteome Extraction**
    1. Resuspend cell pellets in 20 mM EPPS buffer (pH 8.0)
    2. Lyse cells using 5 freeze-thaw cycles in liquid nitrogen
    3. Clear lysate by centrifugation at 14,000 g for 10 min
    4. Determine protein concentration using the BCA assay
    5. Aliquot 0.5 mL of lysate at 1 mg/mL protein concentration
3. **Drug Treatment**
    1. Divide lysate samples into treatment groups
    2. Incubate with either vehicle (DMSO) or test compound (10 μM) for 30 min at 25°C
    3. Prepare at least 3 biological replicates per condition
4. **Trypsin Digestion**
    1. Add trypsin at 1:100 (w/w) ratio to all samples
    2. Begin digestion at 25°C
    3. For each hour (1-8 hours):
        1. Load samples onto 3 kDa MWCO filters
        2. Centrifuge at 14,000 g for 10 min
        3. Collect the filtrate containing digested peptides
        4. Add fresh buffer to the undigested proteins above filter
        5. Continue digestion
5. **TMT Labeling**
    1. Add TMT16 reagents at a 4× weight ratio to each timepoint sample
    2. Incubate for 2 hours at room temperature
    3. Quench reaction with 0.5% hydroxylamine (final concentration), incubate for 15 minutes

6. **Sample Pooling and Reduction/Alkylation**
    1. Combine all timepoint samples within each replicate, put samples in Speedvac, and dry to 50% of the initial concentration
    2. Add DTT to a 10 mM final concentration, incubate 1 hour at RT
    3. Add IAA to a 50 mM final concentration, incubate 1 hour at RT in the dark
    4. Acidify with TFA to pH 2-3
7. **Sample Cleanup and Fractionation**
    1. Clean samples using Sep-Pak cartridges
    2. Dry using SpeedVac concentrator
    3. Resuspend in 20 mM ammonium hydroxide
    4. Fractionate into 96 fractions using an XBridge BEH C18 column:
        1. Gradient: 1-63% B over 48 min (B = 20 mM NH_4OH in ACN)
        2. Flow rate: 200 µL/min
    5. Concatenate fractions into 24 samples sequentially
    6. Resuspend each fraction in 0.1% FA to 1 µg/µL
8. **Dose-Response Experiment** (if performing conc-AFDIP)
    1. Aliquot lysate into 18 tubes at 1 mg/mL protein
    2. Treat with compound concentration series (0-1000 nM) for 30 min at 25°C
    3. Add trypsin (1:100 ratio), digest for 2 hours at 25°C
    4. Stop digestion with aprotinin
    5. Proceed with TMT labeling as above
9. **Chromatographic Separation**
    1. Load ~1 µg sample onto a 50 cm EASY-Spray column
    2. Use 150-min gradient: 3-95% Buffer B at 300 nL/min
    3. Buffer A: 0.1% FA, 2% ACN, 98% H_2O
    4. Buffer B: 98% ACN, 0.1% FA, 2% H_2O
10. **Mass Spectrometry**
    1. Acquire spectra using Orbitrap Fusion Lumos in DDA mode
    2. MS resolution: 120,000 (m/z 375-1500)
    3. MS/MS resolution: 45,000
    4. Fragmentation: HCD with normalized energy 33%

Data Processing and Analysis

11. **MaxQuant Analysis**
    1. Process raw data with MaxQuant v1.5.3.8
    2. Search against human IPI database (2014_02, 89,054 entries)
    3. Fixed modification: Cysteine carbamidomethylation
    4. Variable modification: N,Q-deamidation
    5. Enzyme: Trypsin/P
    6. Maximum missed cleavages: 2
12. **Center of Gravity Calculation**
    1. For each peptide, calculate the Center of Gravity (CoG):
    2. $CoG = \Sigma(ti \times ai) / \Sigma(ai)$

Where ti = timepoint (hours 1-8), ai = normalized abundance

    3. Calculate $\Delta CoG = CoG(treated) - CoG(control)$
    4. Perform statistical analysis using Student's t-test ($p < 0.05$)
13. **Target Protein Identification**
    1. Combine p-values for peptides from the same protein using Fisher's method:
    2. $X^2 2k = -2\Sigma \ln(pi)$
    3. Apply Bonferroni correction for multiple hypothesis testing
    4. Generate volcano plots (ΔCoG vs -log10(p-value))
14. **Binding Site Analysis**
    1. Select the top 3 peptides with the most significant shifts per target protein
    2. Calculate the center of mass (CoM) of selected peptides
    3. Compare with known binding sites from crystal structures
    4. Expected resolution: ≤10 Å (≤5 Å for larger proteins)