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Quantitative Irreversible Tethering (qIT) for Target-directed Covalent Fragment Screening

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[Abstract] Small molecules that react to form covalent bonds with proteins are widely used as biological tools and therapeutic agents. Screening cysteine-reactive fragments against a protein target is an efficient way to identify chemical starting points for covalent probe development. Mass spectrometry is often used to identify the site and degree of covalent fragment binding. However, robust hit identification requires characterization of the kinetics of covalent binding that can be readily achieved using quantitative irreversible tethering. This screening platform uses a non-specific cysteine-reactive fluorogenic probe to monitor the rate of reaction between covalent fragments and cysteine containing biomolecules. Fragment libraries are simultaneously screened against the target protein and glutathione, which functions as a control, to identify hit fragments with kinetic selectivity for covalent modification of the target. Screening by quantitative irreversible tethering accounts for variations in the intrinsic reactivity of individual fragments enabling robust hit identification and ranking.

Keywords: Covalent fragments, Irreversible inhibition, Quantitative irreversible tethering, Fragment-based drug discovery, Cysteine targeting

[Background] Site-directed ligand discovery was first reported in 2000 and utilizes surface-exposed cysteine residues to covalently trap disulfide-linked fragments that bind at an adjacent pocket (Erlanson *et al.*, 2000). Since then, irreversible cysteine-targeting inhibitors have grown immensely in popularity and now represent front-line therapies in multiple oncology indications, with notable examples targeting BTK and EGFR as well as previously undruggable targets such as KRAS (G12C). Following these developments, covalent fragment-based ligand discovery has emerged as an efficient route towards the design of target-specific covalent inhibitors (Resnick *et al.*, 2019). Libraries of cysteine-reactive fragments are now commercially available from a range of compound vendors and utilize a variety of reaction types for covalent bond formation such as conjugate addition and nucleophilic substitution. Protein mass spectrometry was adopted early on as a useful assay for conducting covalent fragment screening, providing a direct readout on the extent and the site of fragment binding. However, even the earliest reports of covalent fragment-based ligand discovery emphasized the challenge that fragment-dependent electrophilicity presents (Nonoo *et al.*, 2012): the intrinsic reactivity of covalent fragments can vary by several orders of magnitude within a library. Fragments with high intrinsic reactivity will covalently bind to proteins regardless of their structure and this reactivity-driven binding does not constitute a useful starting point for fragment optimization. In pioneering work by Statsyuk, it was

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demonstrated that, through careful design of the reactive warhead architecture, it is possible to limit the variations in intrinsic reactivity, resulting in more robust screening outcomes (Kathman *et al.*, 2014). However, in many cases it is desirable to screen a wide variety of warhead structures, maximizing the exploration of chemical space and enlarging the pool of accessible targets (Keserű *et al.*, 2016).

Quantitative irreversible tethering (qIT) is a platform for screening cysteine-reactive fragments that has been designed to overcome the intrinsic reactivity problem (Craven *et al.*, 2018). The fluoregenic probe CPM (7-diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin) is used to quantify the rate of reaction between covalent fragments and cysteine residues. Covalent fragments are simultaneously screened against glutathione (GSH) as well as the target protein and the rates of reactions (k_{obs}) determined in high-throughput. GSH functions as a control cysteine containing biomolecule that lacks significant secondary structure. The rate of reaction between a fragment and GSH represents a measure of the covalent fragment's intrinsic reactivity. Hit fragments are those that react significantly faster with the target protein than with GSH, as quantified by the rate enhancement factor (REF = k_{obs}(target)/k_{obs}(GSH). Screening by REF analysis ensures that qIT can be carried out in high-throughput and does not suffer from high false-positive/negative hit rates because it accounts for intrinsic fragment reactivity. The key advantage of screening by quantitative irreversible tethering is that, in accounting for intrinsic fragment reactivity, true hits can be readily distinguished from non-selective reactive molecules which otherwise lead to high levels of false positives. The platform is compatible with a broad range of cysteine-reactive fragments and is applicable to most cysteine-containing soluble protein domains. Additionally, the high-throughput nature and low cost of using a plate-based fluorescence readout means that quantitative irreversible tethering projects can be performed rapidly and with high efficiency.

Since the original publication of qIT (Craven *et al.*, 2018), we have updated the screening protocol to make it more applicable to a wider variety of proteins by changing the buffer system, more high-throughput by reformatting from 96-well to 384-well format and easier to implement by changing the environment of operation from 4 °C to room temperature. In our lab we have successfully applied this updated protocol to a range of protein classes including kinases, GTPases, oxidoreductase and proteases.

Materials and Reagents

1. Black 384-well plates (Corning, 384-well low flange black flat bottom NBS, catalog number: 3575)
2. Pippette tips for pipetting station (CyBio, SELMA 384/25 µl pipette tips (standard), catalog number: OL3800-25-513-N)
3. Plate sealing tape (Bio-Rad, optical sealing tape, catalog number: 2239444)
4. 384-Well compound storage plates (Nunc, catalog number: 269390)
5. PD-10 column (GE Healthcare, catalog number: 52130800)
6. 15 ml Centrifuge tubes (Star Lab, Catalog number: E1415-0200)
7. Reagent reservoirs (Thermo Scientific, catalog number: 8096-11)

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8. Immobilized TCEP agarose (Pierce, catalog number: 77712). Store at 4 °C (shelf-life ~1 month, soluble TCEP can be added to a final concentration of 50 µM to extend the shelf-life)
9. CPM: 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (Thermofisher, catalog number: D346). Store at 0.5 mM in DMSO at -20 °C in aliquots of 56 µl
10. L-Glutathione reduced (Sigma-Aldrich, catalog number: G4251). Store at 4 °C
11. Target protein–containing a single surface-exposed/reactive cysteine residue (cloning and expression by standard molecular biology protocols as required). See Note 1 for compatibility considerations
12. Control protein–this should be identical in sequence to the target protein but have the reactive cysteine knocked-out (cloning and expression by standard molecular biology protocols as required). Typically, the reactive cysteine is mutated to alanine or serine
13. Covalent fragment library (Compounds stored at 50 mM in DMSO in 384-well compound storage plates, 320 compounds per plate in columns 3-22, columns 1, 2, 23 and 24 are blank). See Note 2 for composition and storage considerations
14. Iodoacetamide (Sigma-Aldrich, catalog number: I1149)
15. HEPES (Sigma-Aldrich, catalog number: H3375)
16. Sodium chloride (Sigma-Aldrich, catalog number: S7653)
17. (Optional) Argon gas (BOC, catalog number: 11Y)
18. DMSO (Sigma-Aldrich, catalog number: D8418)
19. Reaction buffer (see Recipes)
20. Quench buffer (see Recipes)
21. CPM Quench solution (see Recipes)
22. 3x TCEP agarose stock (1.5% suspension) (see Recipes)
23. 3x GSH stock (15 µM) (see Recipes)
24. 3x Target protein stock (15 µM) (see Recipes)
25. 3x Control protein stock (15 µM) (see Recipes)

Equipment

1. Timer (Lab Alert, catalog number: HS24670)
2. Single channel pipettes (Gilson)
3. 12-Channel pipette (Sartorius, Picus electronic 10-300 µl, catalog number: 735461)
4. Plate reader (BMG LABTECH, model: CLARIOstar)
5. Semi-automated pipetting station (Analytik Jena, model: CyBio SELMA 384/25 µl)
6. Centrifuge with plate adapter (Ependorf, model: 5804R)
7. NanoDrop spectrophotometer (Thermo scientific, catalog number: ND-2000)

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Software

1. Microsoft Excel
2. GraphPad Prism 8

Procedure

A. Pilot assay to assess protein construct compatibility and robustness

1. Assemble the reaction plate (see Table 1 for layout).

Table 1. Reaction plate layout for the pilot assay

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
B	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
C	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
D	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
E	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
F	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
G	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
H	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
I	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
J	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
K	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
L	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
M	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
N	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
O	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA
P	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA	B+D	G+D	TP+D	CP+D	B+IA	G+IA	TP+IA	CP+IA

B = buffer; G = GSH; TP = target protein; CP = control protein; D = DMSO; IA = iodoacetamide

   a. Take a black 384-well plate and label it “Reaction plate”
   b. Dispense 20 µl of freshly prepared 3x TCEP agarose stock from a reagent reservoir into each well of the reaction plate using a 12-channel pipette. During pipetting, the beads require regular agitation in the reservoir to maintain a homogeneous suspension and the bottoms of the pipette tips should be cut off to prevent them from blocking.
   c. Dispense 20 µl of reaction buffer into columns 1, 5, 9, 13, 17 and 21 of the reaction plate from a reagent reservoir using a 12-channel pipette.
   d. Dispense 20 µl of 3x GSH stock into columns 2, 6, 10, 14, 18 and 22 of the reaction plate from a reagent reservoir using a 12-channel pipette.
   e. Dispense 20 µl of 3x target protein stock into columns 3, 7, 11, 15, 19 and 23 of the reaction plate from a reagent reservoir using a 12-channel pipette.
   f. Dispense 20 µl of 3x control protein stock into columns 4, 8, 12, 16, 20 and 24 into the reaction plate from a reagent reservoir using a 12-channel pipette.
   g. Incubate at room temperature (RT) for 2 h to ensure complete reduction of cysteine residues by TCEP agarose.
2. Initiate the iodoacetamide and DMSO control reactions.
   a. Dispense 20 µl of reaction buffer containing 3% DMSO into columns 1, 2, 3, 4, 9, 10, 11, 12, 17, 18, 19 and 20 of the reaction plate from a reagent reservoir using a 12-channel pipette.

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b. Dispense 20 µl of 1.5 mM iodoacetamide in reaction buffer containing 3% DMSO into columns 5, 6, 7, 8, 13, 14, 15, 16, 21, 22, 23 and 24 from a reagent reservoir using a 12-channel pipette.
c. Mix all wells by aspirating and dispensing 25 µl three-times using the 384 well semi-automated pipetting station (for details of how to operate the pipetting station please refer to www.analytik-jena.com/products/liquid-handling-automation/flexible-benchtop-automation/cybio-selma/).
d. Start a timer.
e. Centrifuge the plate at 200 *x g* for 3 min to pellet the TCEP agarose.

3. Perform the first CPM quench (Q1).
   a. Take a new black 384-well plate and label it “Quench plate Q1”.
   b. Prepare 20 ml of fresh CPM quench solution (see Recipes).
   c. Dispense 27 µl of CPM quench solution into each well of quench plate Q1.
   d. Using the 384 well semi-automated pipetting station aspirate 3 µl from the top of each well of the reaction plate and dispense into quench plate Q1 (aspirating from the top ensures that no TCEP agarose is transferred).
   e. Mix all wells of the quench plate by aspirating and dispensing 25 µl three-times.
   f. Record the time on the timer (this should be between 5 and 15 min).
   g. Seal the reaction plate with plate sealing tape to prevent evaporation and store at RT until the next quench.
   h. Incubate the quench plate Q1 for 1 h at RT.
   i. Measure the fluorescence intensity of quench plate Q1 (excitation/emission: 384/470 nm).
4. Perform the second CPM quench (Q2) by repeating Step A3 when the timer is at 1 h.
5. Perform the third CPM quench (Q3) by repeating Step A3 when the timer is at 5 h.
6. Perform the fourth CPM quench (Q4) by repeating Step A3 when the timer is at 24 h.
7. After the final measurement export the data into Microsoft Excel for data processing.

B. Covalent fragment screening of 320 compounds (see Figure 1 for schematic workflow)

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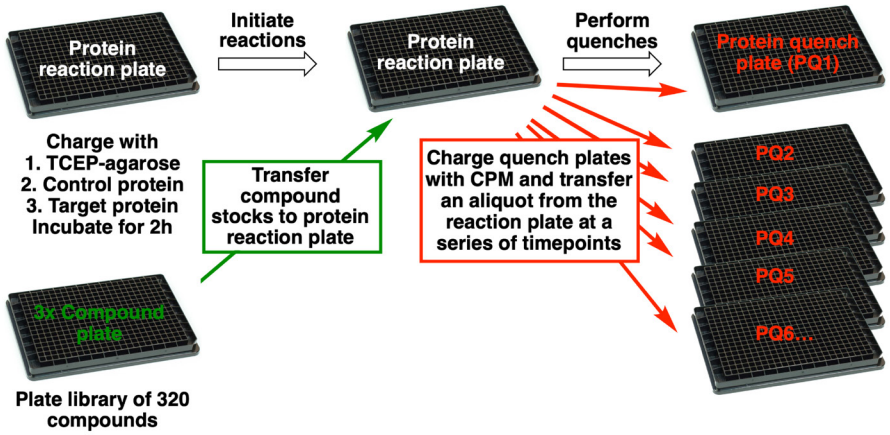

Figure 1. Schematic workflow for covalent fragment screening by qIT

1. Assemble the protein reaction plate.
   a. Take a black 384-well plate and label it “Protein reaction plate”.
   b. Dispense 20 µl of freshly prepared 3x TCEP agarose stock from a reagent reservoir into each well of the protein reaction plate using a 12-channel pipette. During pipetting, the beads require regular agitation in the reservoir to maintain a homogeneous suspension and the bottoms of the pipette tips should be cut off to prevent them from blocking.
   c. Dispense 20 µl of 3x control protein stock into columns 1 and 2 of the protein reaction plate from a reagent reservoir using a 12-channel pipette.
   d. Dispense 20 µl of 3x target protein stock into columns 3-24 of the protein reaction plate from a reagent reservoir using a 12-channel pipette.
   e. Incubate at RT for 2 h to ensure complete reduction of cysteine residues by TCEP agarose.
2. Assemble the GSH reaction plate.
   a. Take a new black 384-well plate and label it “GSH reaction plate”.
   b. Dispense 20 µl of freshly prepared 3x TCEP agarose stock from a reagent reservoir into each well of the protein reaction plate using a 12-channel pipette. During pipetting, the beads require regular agitation in the reservoir to maintain a homogeneous suspension and the bottoms of the pipette tips should be cut off to prevent them from blocking.
   c. Dispense 20 µl of reaction buffer into columns 1 and 2 of the GSH reaction plate from a reagent reservoir using a 12-channel pipette.
   d. Dispense 20 µl of 3x GSH stock into columns 3-24 of the GSH reaction plate from a reagent reservoir using a 12-channel pipette.
   e. Incubate at RT for 2 h to ensure complete reduction of GSH by TCEP agarose.
3. Assemble the 3x compound stock plate.
   a. Take a new black 384-well plate and label it “3x compound plate”.
   b. Dispense 58.2 µl of reaction buffer into all wells of the 3x compound plate from a reagent reservoir using a 12-channel pipette.

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c. Dispense 1.8 µl of DMSO into columns 1, 2, 23, and 24 of the 3x compound plate using the semi-automated pipetting station.
d. Using the semi-automated pipetting station aspirate 1.8 µl from each well of the covalent fragment library storage plate (compounds are in columns 3-22, columns 1, 2, 23 and 24 are blank) and dispense into the 3x compound plate.
e. Mix all wells of the 3x compound plate by aspirating and dispensing 25 µl three-times.
f. Centrifuge the 3x compound plate at 200 *x g* for 3 min to pellet any precipitates.
g. Seal and store the covalent fragment library storage plate.

4. Initiate the protein reactions (see Table 2 for final layout).

Table 2. Protein reaction plate layout for the covalent fragment screen

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	CP+D	CP+D	TP+F1	TP+F2	TP+F3	TP+F4	TP+F5	TP+F6	TP+F7	TP+F8	TP+F9	TP+F10	TP+F11	TP+F12	TP+F13	TP+F14	TP+F15	TP+F16	TP+F17	TP+F18	TP+F19	TP+F20	TP+D	TP+D
B	CP+D	CP+D	TP+F21	TP+F22	TP+F23	TP+F24	TP+F25	TP+F26	TP+F27	TP+F28	TP+F29	TP+F30	TP+F31	TP+F32	TP+F33	TP+F34	TP+F35	TP+F36	TP+F37	TP+F38	TP+F39	TP+F40	TP+D	TP+D
C	CP+D	CP+D	TP+F41	TP+F42	TP+F43	TP+F44	TP+F45	TP+F46	TP+F47	TP+F48	TP+F49	TP+F50	TP+F51	TP+F52	TP+F53	TP+F54	TP+F55	TP+F56	TP+F57	TP+F58	TP+F59	TP+F60	TP+D	TP+D
D	CP+D	CP+D	TP+F61	TP+F62	TP+F63	TP+F64	TP+F65	TP+F66	TP+F67	TP+F68	TP+F69	TP+F70	TP+F71	TP+F72	TP+F73	TP+F74	TP+F75	TP+F76	TP+F77	TP+F78	TP+F79	TP+F80	TP+D	TP+D
E	CP+D	CP+D	TP+F81	TP+F82	TP+F83	TP+F84	TP+F85	TP+F86	TP+F87	TP+F88	TP+F89	TP+F90	TP+F91	TP+F92	TP+F93	TP+F94	TP+F95	TP+F96	TP+F97	TP+F98	TP+F99	TP+F100	TP+D	TP+D
F	CP+D	CP+D	TP+F101	TP+F102	TP+F103	TP+F104	TP+F105	TP+F106	TP+F107	TP+F108	TP+F109	TP+F110	TP+F111	TP+F112	TP+F113	TP+F114	TP+F115	TP+F116	TP+F117	TP+F118	TP+F119	TP+F120	TP+D	TP+D
G	CP+D	CP+D	TP+F121	TP+F122	TP+F123	TP+F124	TP+F125	TP+F126	TP+F127	TP+F128	TP+F129	TP+F130	TP+F131	TP+F132	TP+F133	TP+F134	TP+F135	TP+F136	TP+F137	TP+F138	TP+F139	TP+F140	TP+D	TP+D
H	CP+D	CP+D	TP+F141	TP+F142	TP+F143	TP+F144	TP+F145	TP+F146	TP+F147	TP+F148	TP+F149	TP+F150	TP+F151	TP+F152	TP+F153	TP+F154	TP+F155	TP+F156	TP+F157	TP+F158	TP+F159	TP+F160	TP+D	TP+D
I	CP+D	CP+D	TP+F161	TP+F162	TP+F163	TP+F164	TP+F165	TP+F166	TP+F167	TP+F168	TP+F169	TP+F170	TP+F171	TP+F172	TP+F173	TP+F174	TP+F175	TP+F176	TP+F177	TP+F178	TP+F179	TP+F180	TP+D	TP+D
J	CP+D	CP+D	TP+F181	TP+F182	TP+F183	TP+F184	TP+F185	TP+F186	TP+F187	TP+F188	TP+F189	TP+F190	TP+F191	TP+F192	TP+F193	TP+F194	TP+F195	TP+F196	TP+F197	TP+F198	TP+F199	TP+F200	TP+D	TP+D
K	CP+D	CP+D	TP+F201	TP+F202	TP+F203	TP+F204	TP+F205	TP+F206	TP+F207	TP+F208	TP+F209	TP+F210	TP+F211	TP+F212	TP+F213	TP+F214	TP+F215	TP+F216	TP+F217	TP+F218	TP+F219	TP+F220	TP+D	TP+D
L	CP+D	CP+D	TP+F221	TP+F222	TP+F223	TP+F224	TP+F225	TP+F226	TP+F227	TP+F228	TP+F229	TP+F230	TP+F231	TP+F232	TP+F233	TP+F234	TP+F235	TP+F236	TP+F237	TP+F238	TP+F239	TP+F240	TP+D	TP+D
M	CP+D	CP+D	TP+F241	TP+F242	TP+F243	TP+F244	TP+F245	TP+F246	TP+F247	TP+F248	TP+F249	TP+F250	TP+F251	TP+F252	TP+F253	TP+F254	TP+F255	TP+F256	TP+F257	TP+F258	TP+F259	TP+F260	TP+D	TP+D
N	CP+D	CP+D	TP+F261	TP+F262	TP+F263	TP+F264	TP+F265	TP+F266	TP+F267	TP+F268	TP+F269	TP+F270	TP+F271	TP+F272	TP+F273	TP+F274	TP+F275	TP+F276	TP+F277	TP+F278	TP+F279	TP+F280	TP+D	TP+D
O	CP+D	CP+D	TP+F281	TP+F282	TP+F283	TP+F284	TP+F285	TP+F286	TP+F287	TP+F288	TP+F289	TP+F290	TP+F291	TP+F292	TP+F293	TP+F294	TP+F295	TP+F296	TP+F297	TP+F298	TP+F299	TP+F300	TP+D	TP+D
P	CP+D	CP + D	TP+F301	TP+F302	TP+F303	TP+F304	TP+F305	TP+F306	TP+F307	TP+F308	TP+F309	TP+F310	TP+F311	TP+F312	TP+F313	TP+F314	TP+F315	TP+F316	TP+F317	TP+F318	TP+F319	TP+F320	TP+D	TP+D

TP = target protein; CP = control protein; D = DMSO; F = Covalent fragment

a. Aspirate 20 µl from each well of the 3x compound plate and dispense into the protein reaction plate using the semi-automated pipetting station.
b. Mix all wells of the protein reaction plate by aspirating and dispensing 25 µl three-times.
c. Start a timer (protein reaction timer).
d. Centrifuge the protein reaction plate at 200 *x g* for 3 min to pellet the TCEP agarose.

5. Initiate the GSH reactions.
   a. Aspirate 20 µl from each well of the 3x compound plate and dispense into the GSH reaction plate using the semi-automated pipetting station.
   b. Mix all wells of the GSH reaction plate by aspirating and dispensing 25 µl three-times.
   c. Start a timer (GSH reaction timer).
   d. Centrifuge the GSH reaction plate at 200 *x g* for 3 min to pellet the TCEP agarose.
6. Perform the first protein CPM quench (PQ1) and GSH CPM quench (GQ1).
   a. Take two new black 384-well plates and label one “Protein quench plate PQ1” and the other “GSH quench plate GQ1”.
   b. Prepare 20 ml of fresh CPM quench solution (see Recipes).
   c. Dispense 27 µl of CPM quench solution into each well of quench plates PQ1 and GQ1.
   d. Using the 384 well semi-automated pipetting station aspirate 3 µl from the top of each well of the protein reaction plate and dispense into quench plate PQ1.

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e. Mix all wells of the PQ1 by aspirating and dispensing 25 µl three-times.
f. Record the time on the protein timer (this should be between 5 and 15 min).
g. Seal the protein reaction plate with plate sealing tape to prevent evaporation and store at RT until the next quench.
h. Using the 384 well semi-automated pipetting station aspirate 3 µl from the top of each well of the GSH reaction plate and dispense into quench plate GQ1.
i. Mix all wells of the GQ1 plate by aspirating and dispensing 25 µl three-times.
j. Record the time on the GSH timer (this should be between 5 and 15 min).
k. Seal the GSH reaction plate with plate sealing tape to prevent evaporation and store at RT until the next quench.
l. Incubate the quench plates PQ1 and GQ1 for 1 h at RT.
m. Measure the fluorescence intensity of the Q1 quench plates (excitation/emission: 384/470 nm).

7. Perform subsequent CPM quenches (Q2-Q9) by repeating Step B6 when the timers are at 0.5 h, 1 h, 2 h, 4 h, 6 h, 18 h, 22 h and 26 h.
8. After the final measurement export the data into Microsoft Excel for data processing.

Data analysis

A. Analysis of pilot assay to assess protein construct compatibility and robustness
   1. Analysis of quench Q1
      a. In Microsoft Excel, calculate the mean and standard deviation for the fluorescence intensity of “Buffer + DMSO” (columns 1, 9 and 17), “Buffer + Iodoacetamide” (columns 5, 13 and 21), “GSH + DMSO” (columns 2, 10, and 18), “GSH + Iodoacetamide” (columns 6, 14, and 22), “Target protein + DMSO” (columns 3, 11, and 19), “Target protein + Iodoacetamide” (columns 7, 15, and 23), “Control protein + DMSO” (columns 4, 12, and 20) and “Control protein + Iodoacetamide” (columns 8, 16 and 24).
      b. Calculate the Z-factor: $Z = 1 - (3(\sigma p + \sigma n)/|\mu p - \mu n|$ (σp = standard deviation of positives, σn = standard deviation of negatives, μp = mean of positives, μn = mean of negatives) for the following combinations:
         i. Positive = “GSH + DMSO”; negative = “Buffer + DMSO”
         ii. Positive = “GSH + DMSO”; negative = “GSH + Iodoacetamide”
         iii. Positive = “Target protein + DMSO”; negative = “Control protein + DMSO”
         iv. Positive = “Target protein + DMSO”; negative = “Target protein + Iodoacetamide”
      c. To check for edge effects, confirm that the column 1 gives a similar mean fluorescence intensity to columns 9 and 17 and that column 24 gives a similar mean fluorescence intensity to columns 8 and 16. See Note 3 if edge effects are observed.
   2. Repeat the above Z-factor analysis for Q2, Q3 and Q4.

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Interpretation of pilot assay results

In general, for application to small molecule screening a Z-factor > 0.5 means that the assay is considered suitable for screening (Zhang *et al.*, 1999). Because this assay is time-dependent we apply the following criteria.

1. Z-factor (Positive = “GSH + DMSO”; negative = “Buffer + DMSO”) should be > 0.5 for all quenches, with a dynamic range ≥ 5. We often see a slight drop for this Z-factor over time but in our lab it is typically > 0.6 at Q4. If a dramatic drop in the dynamic range and Z-factor is observed over time, this indicates that the TCEP-agarose beads may have oxidized and need replacing.
2. Z-factor (Positive = “GSH + DMSO”; negative = “GSH + Iodoacetamide”) should increase over time and be > 0.5 for Q3 and Q4.
3. Z-factor (Positive = “Target protein + DMSO”; negative = “Control protein + DMSO”) should be > 0.5 for all quenches, with a dynamic range ≥ 10. This indicates that the cysteine of interest on the target protein is reacting successfully with CPM.
4. The mean fluorescence intensity of the “Control protein + DMSO” should also be compared against “Buffer + DMSO”. Here, a dynamic range of up to 2 is typical. But if it is substantially more than 2, this may indicate that there are other cysteines on the construct that are reacting with CPM. If this is the case, the construct should be reevaluated (see Note 1) and additional cysteine mutations may be required.
5. Z-factor (Positive = “Target protein + DMSO”; negative = “Target protein + Iodoacetamide”) should increase over time and be > 0.5 for Q3 and Q4.

B. Covalent fragment screening

1. Normalize the fluorescence intensity values for PQ1 in Microsoft Excel (see Supplementary Excel Sheet for a template including mock data).
   a. Calculate the mean fluorescence intensity of “Control protein + DMSO” (columns 1 and 2).
   b. Calculate the mean fluorescence intensity of “Target protein + DMSO” (columns 23 and 24).
   c. Subtract the mean fluorescence intensity of “Control protein + DMSO” from the mean fluorescence intensity of “Target protein + DMSO” to give the “Baselined maximum signal”.
   d. Subtract the mean fluorescence intensity of “Control protein + DMSO” from the fluorescence intensities of each of the reaction wells (columns 3-22) to give a “Baselined fluorescence intensity” for each reaction.
   e. Divide each of the 320 “Baselined fluorescence intensity” values by the “Baselined maximum signal” to give a “Normalised fluorescence intensity” for each reaction at Time (of quench) = Q1.
2. Normalize the fluorescence intensity values for PQ2-9 as above for PQ1.
3. Create an XY data table titled “Protein kinetics” in GraphPad Prism with Time (of quench) in minutes = X and Normalized fluorescence intensity = Y for each covalent fragment.

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4. Insert: New Analysis: Non-linear regression (curve fit): One Phase Decay with the following restraints: 0 > Y_0 > 0.2; Plateau > 0.8.
5. Insert: New Graph for Existing Dataset: select Create a New Graph for Each Dataset.
6. Check the R squared values for each reaction and highlight any that are low. Note that in the case of very slow or fast reactions, the R squared value will be low and in this case the observed rate constant should not be taken at face value. Manually inspect each graph for outliers. Sometimes one of the individual quenches may exhibit outliers across the whole plate and these should be excluded.
7. Copy the K value for each reaction into a new Microsoft Excel table called “REF Analysis” under the column heading “k_{obs} target protein”.
8. Normalize the fluorescence intensity values for GQ1 in Microsoft Excel
    a. Calculate the mean fluorescence intensity of “Buffer + DMSO” (columns 1 and 2).
    b. Calculate the mean fluorescence intensity of “GSH + DMSO” (columns 23 and 24).
    c. Subtract the mean fluorescence intensity of “Buffer + DMSO” from the mean fluorescence intensity of “GSH + DMSO” to give the “Baselined maximum signal”.
    d. Subtract the mean fluorescence intensity of “Buffer + DMSO” from the fluorescence intensities of each of the reaction wells (columns 3-22) to give a “Baselined fluorescence intensity” for each reaction.
    e. Divide each of the 320 “Baselined fluorescence intensity” values by the “Baselined maximum signal” to give a “Normalised fluorescence intensity” for each reaction at Time (of quench) = Q1.
9. Normalize the fluorescence intensity values for GQ2-9 as above for GQ1.
10. Create an XY data table titled “GSH kinetics” in GraphPad Prism with Time (of quench) in minutes = X and Normalised fluorescence intensity = Y for each covalent fragment.
11. Insert: New Analysis: Non-linear regression (curve fit): One Phase Decay with the following restraints: 0 > Y_0 > 0.2; Plateau > 0.8.
12. Insert: New Graph for Existing Dataset: select Create a New Graph for Each Dataset.
13. Check the R squared values for each reaction and note any that are low. Manually inspect each graph for outliers.
14. Copy the K value for each GSH reaction into the Microsoft Excel table called “REF Analysis” under the column heading “k_{obs} GSH”.
15. Divide the k_{obs} Target protein column by the k_{obs} GSH column to generate a REF for each reaction.
16. Calculate the geometric mean and standard deviation of the REFs.
17. Hit fragments may be defined as those with a REF that is 3 standard deviations over the geometric mean or can be ordered by REF and an arbitrary hit rate defined (*e.g.*, top 2%). The protein and GSH reaction graphs in Prism should then be manually inspected for all hit compounds to confirm that the curve fitting and data quality are suitable.

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Follow up from primary covalent fragment screen: All hit compounds from a primary screen should be rescreened in triplicate by the same protocol to confirm reproducibility and for reliable quantitative hit ranking. It may also be desirable to rescreen hits at multiple concentrations (*e.g.*, 500, 250, 100 and 50 μM) to gain further insights into the concentration dependency of the modifications. Where possible, hits should then be orthogonally validated by mass spectrometry, biochemical assays and/or X-ray crystallography. For an example of how to conduct hit-follow up please see our recent publication on qIT (Craven *et al.*, 2020).

Notes

1. Where possible, the structure of the target protein should be analyzed to identify any cysteine residues that are likely to be exposed to solvent. In cases where the target has more than one solvent-exposed cysteine residue, site-directed mutagenesis should be applied to generate a construct containing only the target cysteine residue on the surface. Point-mutations can affect the structure or biochemical activity of the target protein and new constructs should be compared against the wild-type to check this has not taken place. Purification tags should also be free from cysteine residues or cleaved off. We generally recommend including reducing agents (such as DTT) in protein purification and storage buffers; however, these reducing agents must be fully removed prior to assay initiation, which can be readily achieved by buffer exchange using a PD-10 column.
2. Commercial vendors of cysteine-targeted covalent fragments include Enamine, LifeChemicals and Cominnex. A review of considerations for covalent fragment library design can also be found (Keeley *et al.*, 2020). Covalent fragment libraries should be subject to ongoing quality control. Storage in D_6-DMSO enables periodic assessment of purity and degradation by NMR. If possible, freeze thaw cycles of DMSO stocks should be avoided minimizing the introduction of atmospheric water.
3. Edge effects typically arise from evaporation or temperature differences across the plate. We do not observe any edge effects in this protocol. But if they are observed, then care should be taken to minimize evaporation by sealing plates carefully when not in use and to ensure that all components are kept at room temperature. If edge effects are still observed, then the plate layout can be redesigned to exclude the use of the outside wells of the plate.

Recipes

1. Reaction buffer
   25 mM HEPES (pH 8.0)
   150 mM NaCl
2. Quench buffer
   25 mM HEPES (pH 7.5)

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150 mM NaCl

Notes:

a. *Additional buffer components may be added to either the reaction buffer and/or quench buffer if required by the target protein for stability e.g., salts and glycerol. However, soluble reducing agents such as DTT, βME or TCEP must not be added as they will react with CPM. The addition of any new buffer components (particularly detergents) should always be tested for compatibility with CPM.*

b. *(Optional) In cases where proteins are highly sensitive to aerobic oxidation, buffers should be degassed by bubbling argon for 30 min prior to use.*

3. CPM Quench solution

   To prepare 20 ml of 1.39 µM CPM quench solution, add 55.6 µl of 0.5 mM CPM in DMSO to 20 ml of quench buffer. A 56 µl aliquot of 0.5 mM CPM in DMSO should be thawed immediately prior to diluting into the quench buffer and the quench should be performed with 15 min of preparation. CPM has low stability at room temperature in water and the performance of the assay is maximized by adhering to this practice.

4. 3x TCEP agarose stock (1.5% suspension)
   a. Pipette 300 µl of immobilized TCEP agarose (stored as a 50% suspension) into a 15 ml centrifuge tube and dilute to 15 ml with reaction buffer
   b. Mix by manual inversion
   c. Centrifuge at 600 *x g* for 1 min to pellet beads
   d. Aspirate and discard the supernatant
   e. Repeat the dilution, mixing, centrifugation and aspirating sequence an additional 4 times
   f. Dilute to 10 ml with reaction buffer

5. 3x GSH stock (15 µM)
   a. Prepare fresh immediately before use
   b. Dissolve 46 mg of reduced glutathione in 10 ml reaction buffer to give a 15 mM stock
   c. Add 15 µl of the 15 mM stock into 15 ml of reaction buffer to give a 15 µM stock

6. 3x Target protein stock (15 µM)
   a. Buffer exchange target protein into reaction buffer using a PD-10 column
   b. Determine the protein concentration by NanoDrop and dilute to 15 µM in reaction buffer
   c. Centrifuge at 2,400 *x g* for 5 min to remove precipitants

7. 3x Control protein stock (15 µM)
   a. Buffer exchange the cysteine-knockout analogue of the target protein into reaction buffer using a PD-10 column
   b. Determine the protein concentration by NanoDrop and dilute to 15 µM in reaction buffer
   c. Centrifuge at 2,400 *x g* for 5 min to remove precipitants

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Competing interests

G.B.C., A.A., and D.J.M. are co-inventors on a patent application covering qIT: PCT/GB2017/052456.

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