Supplemental Information

Seamless Bead to Microarray Screening:

Rapid Identification of the Highest Affinity

Protein Ligands from Large Combinatorial Libraries

John M. Astle, Levi S. Simpson, Yong Huang, M. Muralidhar Reddy, Rosemary Wilson, Steven Connell, Johnnie Wilson, and Thomas Kodadek
Figure S1.
Representative MS and MSMS spectra for FLAG peptide cleaved off of individual 75μm TentaGel beads using the (A) Nffα-Met linker, (B) Nffα-Asp-Pro linker, or (C) Cys-Asp-Pro linker.
Figure S2.
FLAG and Myc peptides were synthesized on the three different linkers and 10 beads from each group were individually cleaved and spotted onto microarrays. The slides were hybridized with anti-FLAG antibody followed by red fluorescently labeled secondary antibodies. Anti-FLAG antibody appears to bind best to spots displaying FLAG peptide that was cleaved and spotted using the Cys-Asp-Pro linker, however, all three linkers appeared to work sufficiently well for microarray analysis. No anti-FLAG antibody binding was detectable on the spots displaying myc peptide.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Synthesis of FLAG peptide and Myc peptide on 75μm TentaGel beads with either Cys-Asp-Pro or Nffα-Met linker

75μm high capacity NH2 tentagel beads (PEG-coated polystyrene beads) from Rapp Polymere (www.rapp-polymere.com catalog # HL12 902) were swelled in DMF overnight and standard FMOC chemistry was used to synthesize FLAG-Cys-Asp-Pro and Myc-Cys-Asp-Pro peptides. 0.2M amino acid, 0.2M HBTU, 0.2M HOBt, 0.4M NMM was used for each amino acid coupling reaction (~1.5 hours per coupling), and 20% piperidine in anhydrous DMF 20 minutes x 2 was used for each FMOC de-protection step. For the FLAG-Nffα-Met and Myc-Nffα-Met compounds, Met was added using the same amino acid coupling reaction described above, while Nffα was added using the peptoid “submonomer” synthesis approach described by Zuckerman and coworkers (reference 14 of the manuscript—see below for detailed description of peptoid and amino acid monomer additions). After Nffα addition, FLAG or Myc peptides were synthesized on the N-terminus following the amino acid addition steps described above. Note: FLAG = Asp-Tyr-Lys-Asp-Asp-Asp-Lys, Myc = Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu.

Protocols for peptoid/D-peptide hybrid library synthesis, magnetic screening, and cleavage for MS sequencing and microarray slide attachment.

ON-BEAD LIBRARY SYNTHESIS
Overview

If you are unfamiliar with split-pool synthesis for one-bead, one-compound library synthesis, read Lam KS, et. al, A new type of synthetic peptide library for identifying ligand-binding activity. Nature. 1991 Nov 7;354(6348):82-4. A (D-amino acid biased) peptoid/D-peptide hybrid library was made on 75μm tentagel beads (~4million beads/g dry beads) using 15 D-amino acids and 5 peptoid monomers. A 6-mer library was made with these 20 monomers, making the theoretical diversity of the library $20^6 = 64$ million. This protocol is written for the Cys-Asp-Pro linker displayed library. For the Nffα-Met linker displayed library, add FMOC-Met-OH then Nffα instead of the other three amino acids (also, use dimethoxybenzylamine instead of furfurylamine for one of the variable peptoid monomers).

Materials

75μm high capacity NH$_2$ tentagel beads (PEG-coated polystyrene beads) from Rapp Polymere (www.rapp-polymere.com catalog # HL12 902) ~4,000,000 beads/g

5ml Disposable Reaction Columns for solid phase synthesis from Intavis AG (www.intavis.com catalog # 99.274)

10ml Disposable Reaction Columns for solid phase synthesis from Intavis AG (www.intavis.com catalog # 99.276)

21G needles (needle should be long enough to reach the bottom of a 15-ml conical when attached to a 5-ml reaction column/vessel—or you’ll have to be tricky about getting the last 0.5ml of liquid out of the bottom of the tube.

Solid Rubber Stoppers (Fisher, catalog # 14130A)

Chemical Hood

Shaker

Scale (accurate in milligram range)

Weigh paper

Spatulas for weighing chemicals

HBTU (EMD/Novabiochem)

HOBt (VWR/Novabiochem)

NMM (N-methylmorpholine) (Sigma)

Piperidine (Sigma, cat # 411027)

Amino Acids (EMD/Novabiochem): FMOC-Pro-OH, FMOC-Asp(OtBu)-OH, FMOC-Cys(Trt)-OH

D-amino acids (EMD/Novabiochem): FMOC-D-Trp(Boc)-OH, FMOC-D-Thr(tBu)-OH, FMOC-D-Ser(tBu)-OH, FMOC-D-Phe-OH, FMOC-D-Leu-OH, FMOC-D-Gly-OH, FMOC-D-Gln(Trt)-OH, FMOC-D-His(Trt)-OH, FMOC-D-Glu(OtBu)-OH, FMOC-D-
Arg(Pbf)-OH, FMOC-D-Asn(Trt)-OH, FMOC-D-Ala-OH, FMOC-D-Lys(Boc)-OH,
FMOC-D-Asp(OtBu)-OH, FMOC-D-Tyr(tBu)-OH

Amines: 4-(2-Aminoethyl)benzenesulfonamide (Sigma), Piperonylamine (Fisher),
Furfurylamine (Fisher), Allylamine (Fisher), cyclohexenylethylamine (Fisher)

BAA (Bromoacetic Acid) (Sigma)

DIC (Diisopropylcarbodiimide) (Sigma)

1-2L Erlenmeyer Flask (for chemical waste)

DMF (N,N-Dimethylformamide) 4x4L (VWR, catalog # EMDX17301) (for swelling and
washing steps)

Anhydrous DMF (anhDMF) Acroseal 1L (Fisher, catalog # AC610320010) (for reaction
steps)

DCM (Dichloromethane) 4L (VWR, catalog # EMDX08381)

Trifluoroacetic Acid 450ml (Applied Biosystems, catalog # 400137)

Triisopropylsilane (Sigma)

MilliQ (filtered, deionized) water

50ml Conical Tubes (Falcon)

15ml Conical Tubes (Falcon)

Sharpie

Squirt Bottle (for DMF)

Pasteur pipettes & bulb(s)

(transfer pipettes)

Something to place 50ml conical tubes in chemical hood

15ml conical tube holder (i.e. styrofoam or cardboard) (for 15ml conicals and to stand
disposable reaction vessels upright during bead splitting steps)

Something that will keep a 50ml conical tube upright on a scale

Something that will keep a 15ml conical tube upright on a scale

Procedure

Day 0

1) Warm 75um beads to room temp (stored at 4°C), label 50ml conical “DMF” (on
side and lid)

2) Use up to 200mg per 5ml reaction vessel (in this case, 20 monomers x 150mg =
3g). Add 0.5g of beads per 10ml reaction vessel (3g/0.5g/vessel = 6 vessels)

3) Place 21G Needle on reaction vessel, in chemical hood, draw up ~8ml DMF from
50ml conical into each reaction vessel (Draw up DMF until plunger hits 10ml
mark, draw in 2ml of air), insert needle tip into rubber stopper, tape the syringes
to a shaker O/N at room temp.

Day 1

4) Warm FMOC-Pro-OH, FMOC-Asp(OtBu)-OH, FMOC-Cys(Trt)-OH, HBTU, and
HOBr to Room Temp (all stored at 4°C). Label three 50-ml conicals as
“Pro,” “Asp,” and “Cys” and three 50-ml tubes as “HHN”

a. 8ml of 0.2M amino acid, 0.2M HBTU, 0.2M HOBr, 0.4M NMM will be
used for each reaction vessel containing 0.5g beads (6 vessels x 8ml +
2ml = 50ml). 0.2M = MW g/mol x 0.2 mol/L = 0.2 x MW g/L = 0.2 x MW
mg/ml. 0.2 x MW mg/ml x 50 ml = (10 x MW) mg per 50ml conical.
  i. Add 3.37g of FMOC-Pro-OH (MW 337.38) to “Pro” tube
  ii. Add 4.11g of FMOC-Asp(OtBu)-OH (MW 411.46) to
“Asp” tube
  iii. Add 5.86g of FMOC-Cys(Trt)-OH (MW 585.72) to “Cys”
tube
  iv. Add 3.79g of HBTU (MW 379.25) to each “HHN” tube
  v. Add 1.35g of HOBr (MW 135.1) to each “HHN” tube

5) Add anhDMF to FMOC-Pro-OH tube: fill up to 25ml mark, shake until
dissolved.

6) Add anhDMF to one of the HHN tubes: fill up to ~22ml mark, shake until
dissolved (some crystals may not dissolve completely—that’s o.k.), place on scale
in something that will keep the 50ml conical tube upright, tare, add 2.04g (a little
more is o.k.) NMM (MW 101.8), add anhDMF up to 25ml, shake until dissolved

7) Combine FMOC-Pro-OH tube and HHN tube, mix

8) Drain DMF from 10ml reaction vessels—there should be an air bubble at the top
so drain all the liquid but leave a little air so you don’t crush the beads. Draw up
the plunger and squeeze down again to get rid of remaining DMF if necessary.
Draw up 8ml of reaction mixture per tube, place in rubber stopper, tape to shaker
2hrs. (optional: do step 14 from day 2 today)

9) Wash beads with DMF by squirting out reaction solution into Erlenmeyer flask
(waste), drawing up DMF from 50ml conical, shaking (~10 sec). Wash 6 times.

10) Add 10ml piperidine to 50ml conical, add up to 50ml anhDMF, label as 20%
piperidine. Draw up 8ml to each tube, leave on shaker 20min @ RT.

11) Repeat step 10. This cleaves the FMOC protecting group from the amino acid.

12) Repeat steps 5-11 for FMOC-Asp(OtBu)-OH, then FMOC-Cys(Trt)-OH. The
FMOC-Cys(Trt)-OH step can be left overnight.

Day 2

13) If FMOC-Cys(Trt)-OH addition was left overnight, cleave FMOC as in steps 10-
11 above.

14) Get 4 50-ml conicals, label two “BAA” and two “DIC” on lid and side. Get 50
15-ml conicals, label 6 of them “HHN”, two “BAA”, two “DIC”, and of the
remaining 40, label two tubes for each of the 20 monomers (i.e. D-ASP, D-Tyr,
Nall, Npip). Half of these will be used today, the rest tomorrow (you can separate
the tubes into two groups of 25 for each day. Weigh out the appropriate amount
of each chemical. 2ml of 0.2M amino acid, 0.2M HBTU, 0.2M HOBr, 0.4M
NMM will be used for each amino acid coupling reaction (~150mg beads per
vessel). Amino acids: 0.2M = MW g/mol x 0.2 mol/L = 0.2 x MW g/L = 0.2 x
MW mg/ml. 0.2 x MW mg/ml x 6 ml = (1.2 x MW) mg per 15ml conical.
HBTU/HOBt will be weighed out into 6 tubes and NMM will be added before
each reaction. 0.2 x MW mg/ml x 15ml = (3 x MW) mg (HBTU and HOBr) per
15ml conical. 0.4 x MW mg/ml x 15ml = (6 x MW) mg NMM will be added per
HHN 15 ml conical. Final concentration of BAA will be 1M, DIC will be 1M, 2ml BAA+DIC per tube (150mg beads). Use ~1-2M amine solutions for peptoid monomers.

b. Add 632 mg FMOC-D-Trp(Boc)-OH (MW 526.59) to “Dtrp” tubes
c. Add 477 mg FMOC-D-Thr(tBu)-OH (MW 397.47) to “Dthr” tubes
d. Add 460 mg FMOC-D-Ser(tBu)-OH (MW 383.45) to “Dserr” tubes
e. Add 465 mg FMOC-D-Phe-OH (MW 387.44) to “Dphe” tubes
f. Add 424 mg FMOC-D-Leu-OH (MW 353.42) to “Dleu” tubes
g. Add 357 mg FMOC-D-Gly-OH (MW 297.31) to “Gly” tubes
h. Add 733 mg FMOC-D-Gln(Trt)-OH (MW 610.71) to “Dgln” tubes
i. Add 744 mg FMOC-D-His(Trt)-OH (MW 619.72) to “Dhis” tubes
j. Add 511 mg FMOC-D-Arg(Pbf)-OH (MW 648.54) to “Darg” tubes
k. Add 779 mg FMOC-D-Asp(OtBu)-OH (MW 596.68) to “Dasn” tubes
l. Add 716 mg FMOC-D-Glu(OtBu)-OH (MW 596.68) to “Dgln” tubes
m. Add 374 mg FMOC-D-Ala-OH (MW 311.34) to “Dala” tubes
n. Add 562 mg FMOC-D-Lys(Boc)-OH (MW 468.55) to “Dlys” tubes
o. Add 494 mg FMOC-D-Asp(OtBu)-OH (MW 411.46) to “Dasp” tubes
p. Add 551 mg FMOC-D-Tyr(tBu)-OH (MW 459.54) to “Dtyr” tubes
q. Add 1.14 g HBTU (MW 379.25) to “HHN” tubes
r. Add 405 mg HOBt (MW 135.1) to “HHN” tubes
s. Add 5.56 g BAA (MW 138.95) to 50ml conical, add up to 20ml anhDMF
t. Add 8ml DIC (MW 126.23) + 12 ml anhDMF in 50ml conical
u. Add 2.5ml Piperonylamine + 3.5ml anhDMF (6ml total) to one “Npip” tube
v. Add 2.5ml Furfurylamine + 3.5ml anhDMF (6ml total) to one “Nffa” tube
w. Add 2.5ml Allylamine + 3.5ml anhDMF (6ml total) to one “Nall” tube
x. Add 2.5ml Cyclohexenylethylamine + 3.5ml anhDMF (6ml total) to one “Nche” tube
y. Weigh out 200mg/ml 4-(2-Aminoethyl)benzenesulfonamide = 1.2g in 6ml anhDMF in one “Nbsa” tube

15) After the last FMOC deprotection, wash beads in DMF 6 times, transfer beads to 20 Intavis 5-ml disposable reaction vessels

z. Get 20 5-ml reaction vessels without plungers, put 21G needle on it, insert into rubber stopper, place in 15-ml conical rack (standing upright, rubber stopper on bottom). Put colored tape around 5 of the 5-ml vessels and use these for peptoid monomer additions (these will be “peptoid vessel” from now on and the non-taped ones will be “amino acid vessels”). Drain DMF from 10ml reaction vessels, suck up about 5-6ml DMF, pull plunger all the way to the top (12ml mark), insert needle into rubber stopper, place 10-ml reaction vessel with rubber stopper into 15ml conical rack (rubber stopper on bottom). Repeat for all 10ml conicals. Pull out plunger from vessel and set aside on a clean, dry surface/paper towel.

aa. Use a transfer pipette to transfer beads from 10-ml reaction vessels to 5-ml reaction vessels. Let beads settle and transfer beads from the vessels with more than average to the vessels with less than average # of beads until all vessels have the same amount of beads in each.

bb. Carefully remove 5-ml syringes from rubber stopper (over the Erlenmeyer waste) and insert plunger. Draw up new DMF into the vessel and set it aside. Repeat for all 20 vessels.

16) Add 3ml anhDMF to all of the tubes with amino acids in them (one per amine—the second tube of each amino acid should be set aside for tomorrow). Vortex until dissolved.

17) Add 12-13ml anhDMF to a “HHN” tube, vortex until HBTU and HOBt are
mostly dissolved, set on scale, tare, add 1.23g (a little more is o.k.) NMM to the tube (drip in in from above the 15-ml conical--do not touch the 15ml conical or you may contaminate the NMM stock). Add anhDMF up to 15ml line, vortex until dissolved.

18) Drain DMF from amino acid vessels (one or two at a time), Draw up 1ml of one of the amino acids (it doesn’t matter which one), touch the side of the needle on a clean, dry paper towel if there is a drop of liquid on it, draw up 1ml from the HHN tube into the same vessel, draw up plunger a little more so there is about a 1-ml air bubble in the vessel (to facilitate shaking), insert needle into rubber stopper, and set aside. Repeat for all amino acid tubes. Wrap all 15 vessels in a paper towel, tape it together, and tape the group onto a shaker snugly so the vessels don’t slip out/off. Leave on shaker 1hr @ RT.

19) Peptoid additions can be done during the one hour incubation or during the first 20-min. FMOC cleavage step after amino acid addition.

cc. Transfer 14 ml BAA and DIC from 50-ml conicals to 15-ml conicals (because these have 1-ml marks on them)

dd. Draw up 1ml from BAA tube and 1ml from DIC tube into peptoid vessel

ee. Draw up plunger until ~1ml air bubble is there, place needle in rubber stopper, remove needle+plunger from vessel, set aside, and repeat for the other four peptoid vessels.

ff. Shake the five vessels by hand until crystals form (about 1 minute)

gg. Place three of the vessels upside down in a glass jar in the microwave

hh. Microwave for 15 sec on power 10 (press “1” then “5” then “power” then “1” then “start”)

ii. Repeat steps d-e for the remaining two vessels, shake the other three by hand while the two are microwaving

jj. Repeat steps d-f (for a total of two 15-sec microwaves per vessel)

kk. Replace needles onto vessels, remove needle from stopper, drain BAA/DIC

ll. Wash with DMF 6 times

mm. Draw up 2 ml of amine per vessel, repeat steps c-h above (except no crystals will form this time)

nn. Wash with DMF 4 times

oo. Drain DMF, draw up about 0.5-1ml DMF, pull plunger all the way to the top, insert needle into rubber stopper, pull plunger completely out, set plunger aside on clean, dry surface/paper towel, and place vessel in 15-ml conical rack.

pp. Label a 50-ml conical tube as “MIX” on the side and lid. Combine the beads with the peptoid monomers on them into this tube by pouring the beads into the 50-ml conical and squirting DMF from a squirt bottle into the vessel while the vessel is mostly upside-down over the 50-ml conical.

20) After the amino acids have coupled for at least 1hr @ RT, DMF wash 5 times, pool the beads into a new 50-ml conical (may need to let beads settle in 50-ml conical and drain excess DMF periodically), redistribute beads into five 10-ml reaction vessels. Wash once with DMF

21) Drain DMF, add ~8ml 20% piperidine to each vessel, shake 20min.

22) Drain, repeat step 21

23) Wash with DMF 5 times, pool into 50-ml conical labeled “MIX” containing beads from peptoid monomer additions. Mix well.

24) Redistribute Beads from “MIX” tube to 20 reaction vessels using transfer pipette.

25) Repeat Steps 17-24 twice.

Day 3

26) Repeat step 14 r-x except only make 15ml of BAA and DIC (4.17g BAA in 15ml
27) Repeat steps 16-25, except after the last monomer addition and pooling beads, leave in “MIX” tube. Beads can be transferred to a 15ml-conical for storage at 4°C if desired. For long-term storage (more than a month or so, wash beads with DCM, air dry overnight, and store at -20°C).

MAGNETIC SCREENING PROTOCOL FOR ON-BEAD LIBRARIES

Overview

On-bead chemical libraries can be quickly and easily screened to identify compounds that will bind specific proteins. Here, the concept is demonstrated using anti-flag antibody as the target protein. Anti-flag antibody will be incubated with the library beads, washed, incubated with magnetic particles conjugated to Sheep-Anti-Mouse IgG antibodies, and beads bound by anti-flag will be pulled out using a magnet. This protocol has been tested on 160um and 75um beads.

Materials

On-bead library (synthesized on 75um high capacity NH₂ tentagel beads (PEG-coated polystyrene beads) from Rapp Polymere (www.rapp-polymere.com catalog # HL12 902)

5ml Disposable Reaction Columns for solid phase synthesis from Intavis AG (www.intavis.com catalog # 99.274)

21G needles

Solid Rubber Stoppers (Fisher, catalog # 14130A)

Rotator (that can rotate/invert 15-ml conical tubes and 5-ml reaction vessels at Room Temperature and at 4°C)

Scale

Weigh paper

Powdered Milk (Carnation Instand Non-Fat Dry Milk)

1-2L Erlenmeyer Flask (for chemical waste)

DMF (N,N-Dimethylformamide) 4x4L (VWR, catalog # EMDX17301) (for swelling and washing steps)

DCM (Dichloromethane) 4L (VWR, catalog # EMDX08381)

Trifluoroacetic Acid 450ml (Applied Biosystems, catalog # 400137)

Triisopropylsilane (Sigma)

MilliQ (filtered, deionized) water

50ml Conical Tubes (Falcon)
15ml Conical Tubes (Falcon)

Sharpie

TBST buffer (have 500mL or more of 1x handy)

DynaMag-15 Magnet (Invitrogen/Dynal Cat. # 123.01D)

For Mouse antibodies, use Dynabeads M-280 sheep anti-mouse IgG (2mL, 6-7X10^8 beads/mL, 10 mg/mL) (Invitrogen Co. Cat # 08099300). 2' antibody-Dynabeads for antibodies from other species are also available.

If screening biotinlyated protein, use Dynabeads M-280 Streptavidin (Invitrogen Co. Cat # 08099300)

Anti-Flag monoclonal (M2) antibody produced in mouse (Sigma Catalog # F3165)

StartingBlock (Fisher Sci. Catalog # 00327600)

Pipetter

5-ml pipettes

Eppendorf Tubes

P1000, P200, P10 pipettemen & tips

Centrifuge for eppendorf tubes

Parafilm

Dissecting microscope

Hand-held rare-earth metal (very strong) magnet (not necessary but helpful to confirm hits). I have a 4x4x1 in. that cost about $100 from a specialty magnet store, but a smaller one should work just as well (smaller ones can be purchased online). Be VERY careful when handling these magnets as they can crush bones, break equipment, erase credit cards, etc.).

Procedure

**Day 1 (library prep)**
If library beads still have protecting groups (if already TFA cleaved and in DMF, skip to step 7):

1) Wash with DCM (in 5- or 10-ml disposable reaction columns) 6-8 times, pull plunger half-way out, tap column to separate beads a little, pull out plunger completely and air dry ~5 minutes.

2) Make enough TFA cocktail for two 30 minute cleavages (~6ml for 5-ml reaction column, ~16ml for 10ml reaction column). TFA cocktail = 95% TFA, 2.5% H2O, 2.5% triisopropylsilane.

3) Insert plunger half way (not all the way or you will crush beads stuck to the side of the column). Draw up some TFA, mix a little (to get the beads wet so the plunger doesn’t crush them), carefully turn column upside-down and push out all
the air, then draw up more TFA (~3ml for 5-ml column, ~8ml for 10ml reaction column). Insert into rubber stopper and place on shaker for 30min.

4) Squirt out TFA into waste, add more TFA, shake for 30 more minutes.

5) DCM wash 8 times

6) DMF wash 8 times, leave in DMF on shaker for at least 1 hour

7) Aliquot the amount of beads you want to screen tomorrow into a 5-ml reaction column and leave the rest in DMF at 4°C (label appropriately—i.e. name, date, library composition, “post-TFA in DMF”)

The following steps are to get rid of any DCM that is stuck inside the porous beads (DMF steps) and then any DMF from the beads (H2O and TBST steps)—longer wash times are o.k. if you want to be extra careful (if transitions from one solution to the next are too quick, beads will stick together and remain stuck). Vortexing may help break apart beads.

8) Squirt out all but 1ml of DMF into chemical waste flask.

9) Add 0.5ml milliQ H2O, shake 20-30 sec.

10) Repeat Step 9 four more times (until solution is ~70% H2O, 30% DMF), leave on shaker 30min at RT.

11) Squirt out all but 1ml DMF, add ~2ml H2O (~90% H2O, 10%DMF), shaker for 30min.

12) Squirt out completely and wash in H2O 6 times, leave in H2O on shaker for at least 1 hour.

13) Wash in TBST once and leave in TBST overnight at 4°C on rotator.

**Day 2 (the screen)**

14) Make 5% milk solution (50mg/ml) in 1:1 TBST:StartingBlock to block non-specific binding of beads.

15) Squirt out TBST, draw up 3ml 5% milk. Leave on rotator at RT 1hr.

16) Make 3ml of 10ug/ml solution of anti-flag antibody in 5% milk in 1:1 TBST:StartingBlock (add 6ul of 5mg/ml anti-flag to 3ml of 5% milk solution).

17) Squirt out 5% milk, remove plunger, place needle in rubber stopper, pour in 3ml anti-flag solution, replace plunger, turn upside down, shake a bit and remove rubber stopper to equilibrate air pressure. Replace rubber stopper and tape to rotator (needle can be removed and column opening covered with parafilm if the column will fit better on the rotator this way). Leave on rotator 1hr @ RT.

18) Squirt out anti-flag solution, wash in TBST 8-10 times by squirting out TBST, drawing more in, inverting ~10 times, repeat.

19) Squirt out TBST and Draw up 3ml of StartingBlock.

20) Transfer beads in startingblock to 15ml conical tube by pulling plunger mostly out, sticking needle in rubber stopper, remove plunger completely, pour into 15-ml conical, pour back and forth to make sure most of the beads are transferred, and if you want to make sure all beads are transferred, use a 1000ul pipetteman.

21) Add 30ul of 10mg/ml Dynabeads M-280 sheep anti-mouse IgG (you can add more startingblock and dynabeads—I use 10ul per ml of solution, typically about 3ml per 500,000 75um beads).

22) Rotate @ RT 20-25 minutes.

23) Add StartingBlock up to 14ml, invert tube until all beads are in suspension, then place tube in DynaMag-15 magnet.

24) Immediately invert the tube inside the magnet (the magnet swivels on a stand) slowly then tilt back and forth for about 2 minutes (to make sure all the beads in the tube have a chance to come in close contact with the sides of the tube where the magnets are).

25) Leave the tube upright, tap the tubes to dislodge any beads held at the top by surface tension, and let all of the non-magnetized beads settle to the bottom of the tube. Your hits should be stuck to the side of the tube and can be visualized (if you have good eyes) by quickly twisting the tube ¼ turns. The brown sandy stuff
is the dynabeads and the slightly bigger beads (if any) are your hits (don’t worry if you can’t see hits at this point—they’re really small).

26) Remove the top 6ml of StartingBlock from your tube with a 5-ml pipette (drain into a new 15-ml conical tube, labeled appropriately). Slide the pipette along the back edge of the 15-ml conical tube to avoid knocking hit beads off of the sides of the tube. Lower your pipette to the bottom of the tube and draw up 6ml, drain in the new 15-ml conical (this will transfer most of the non-magnetized beads). Finally, while slowly tapping the pipette up and down on the bottom of the 15-ml conical, draw up the remaining 2ml and keep drawing up air as you remove the pipette from the tube (this will prevent dripping beads back into the tube—but be careful not to suck liquid into the pipetter!).

27) Immediately add 14ml TBST to the tube, remove tube from the magnet and invert to get all of the beads in suspension (I invert until the brown dynabeads on the sides of the tube are in suspension), insert back into the magnet.

28) Repeat steps 24-27.

29) Repeat steps 24-26.

30) After the last wash, add 1ml TBST to the 15-ml conical. Invert back and forth to get any hit beads and the dynabeads (that are stuck on the side of the tube) suspended in the 1ml of TBST. At this point, if you have good eyes, you can place a hand-held rare-earth metal magnet on the side of the bottom of the tube to see if you have any hit beads that follow the magnet around as you twist the tube, while any “negative” beads will remain at the bottom of the tube. I prefer to transfer these to an eppendorf tube to confirm under the microscope (see next step)

31) Transfer TBST and beads to eppendorf tube with 1000ul pipetteman.

32) Visualize beads under a dissecting microscope. Place a magnet on the side of the tube and all of the dynabeads and hit beads will move to that side of the tube (be EXTREMELY careful as the microscope has metal parts and sometimes tables and surrounding structures have metal—you can hurt yourself, equipment, or the magnet if not careful). Now twist the tube around several times. Sometimes the wall of dynabeads moving to the side of the tube traps “negative” beads there, and twisting the tube around allows these non-magnetized beads to settle to the bottom of the tube, while any hit beads will follow the magnet. If any negative are present, they will settle to the bottom and you can carefully pipette them out with a P200 pipetteman (by keeping your hits on the side of the tube with the magnet and pipetting off the bottom of the tube). Confirm all negatives are removed under the microscope. Note—a single round of magnetic screening may not pull out all of your hits (though most of the best ones should come out first) and your pool of “negatives” can be rescreened by either adding new dynabeads or recycling the old ones after they’re separated from your hits (they appear to be recyclable around two to three times before they stop pulling out beads).

Remove Dynabeads and prep for Spotting onto microarrays and MS sequencing:

33) Invert the tube until all dynabeads are in suspension. Spin in centrifuge briefly (add counterweight tube, either use “shortspin” button that spins when pushed but stops when you let go, you hit spin then stop. I let the speed get up to 2,500 rpm then immediately immediately stop it). This will spin your hits to the bottom of the tube while the dynabeads remain in suspension.

34) Immediately draw off the top ~1ml of TBST, including most of the Dynabeads. ***Make sure you can see all of your hit beads at the bottom of the tube the entire time to ensure you don’t lose any of your hits!!!

35) Add 1ml milliQ H20, spin down as in step 33.

36) Repeat steps 34-35 6-8 times (sometimes your hits will no longer be magnetized after the washes, but don’t worry). The last 2 washes can be done with HPLC/MS grade H20 to minimize background in MS spectra (nice, but not necessary to
CLEAVAGE OF COMPOUNDS OFF OF HIT BEADS WITH THE CYS-ASP-PRO LINKER FOR MS SEQUENCING AND MICROARRAY SLIDE ATTACHMENT

Overview

Compounds containing a Cys-Asp-Pro linker can be cleaved from beads in hot acidic conditions fairly specifically at the amide bond between Asp and Pro. The Cysteine allows subsequent attachment to maleimide-coated microarray slides via a Michael Addition reaction for high-throughput characterization of many hits.

Materials

PCR tubes (I use 12-strip 0.2ml)

10ul, 200ul pipettemen, tips

Parafilm

TFA

Dissecting microscope

Sigmacote (Sigma cat # 00768700) optional

Petri Dish (any size that you feel comfortable working with under dissecting scope)

HPLC/MS grade Acetonitrile

HPLC/MS grade H20

5mg/ml α-Cyano-4-hydroxycinnamic acid in 1:1 Acetonitrile:H20

DMSO

384-well polypropylene plate (i.e. Whatman Microplate Devices Uniplate, 384 wells, 80uL, Polypropylene, V bottom, cat # 7701-5101)

Sticky covers for 384-well plate (i.e. Corning Incorporated costar cat # 6570)

Access to a Tandem TOF/TOF Mass Spectrometer (we have an Applied Biosciences 4700 Proteomics Analyzer) and MSMS plate and someone who can help you use it if you’re not properly trained, as well as cleaning reagent, soft bristled toothbrush, polish, kimwipes, and methanol for cleaning the MSMS plate

Access to a Microarray spotter (we use a TeleChem International NanoPrint LM 360 with MP946 Micro Spotting Pins) and someone who can help you use it if you’re not properly
Access to a PCR machine (we use an MJ Research PTC-200 Peltier Thermal Cycler)

Ethanol

Procedure

1. Under the dissecting microscope, visualize hit beads in your eppendorf tube. Let them settle to the bottom and then pipette them out onto a petri dish.
2. Using a 10ul pipette man, set it to 1ul and pipette up one bead at a time and transfer to the bottom of a PCR tube. Label the tubes appropriately. If beads are difficult to pipette off of the plate, the petri dish can be coated with Sigmacote prior to transferring to PCR tubes. Once you get the hang of this, you should be able to transfer at least 2 beads per minute (getting the hang of it takes some practice).
3. After 12 beads are transferred to a 12-strip of tubes, visualize the bottoms of each tube under the microscope to confirm only one bead per tube was transferred.
4. Once all beads are transferred, add 10ul of 0.12% TFA in H2O (HPLC/MS grade) to each tube.
5. Heat to 95°C for 40 minutes in a PCR machine.
6. Add 10ul of DMSO to one well per hit bead
7. Transfer 10ul of solution from each of the PCR tubes containing cleaved hits to the 384-well plate. Make sure tubes are marked properly and the location of each compound in the 384-well plate are logged properly in your lab book. Cover the plate with a sticky cover or proceed to spotting onto microarray slides
8. Add 8ul of acetonitrile to each of the PCR tubes.
9. Cut a piece of parafilm, fold creases in it with the inside of the creases facing the paper cover. Remove the paper cover.
10. Add 1ul from tube 1 to the first crease in the parafilm.
11. Add 1ul of 5mg/ml α-Cyano-4-hydroxycinnamic acid in 1:1 Acetonitrile:H2O to the 1ul on the parafilm, pipette up and down to mix, and spot ~1ul onto a spot on the MSMS plate.
12. Repeat steps 10-11 for all of the hit bead PCR tubes, and record which tubes correspond to which spot on the MSMS plate.
13. Sequence hits.

CLEAVAGE OF COMPOUNDS OFF OF HIT BEADS WITH THE NFFA-MET LINKER FOR MS SEQUENCING AND MICROARRAY SLIDE ATTACHMENT

Overview

Compounds containing a Nffa-Met linker can be cleaved from beads in CNBr overnight at room temperature (there was also a microwave method recently published for CNBr cleavage of methionine but I haven’t tried it on single 75um beads). The furan-containing Nffa monomer allows subsequent attachment to maleimide-coated microarray slides via a Diels-Alder reaction for high-throughput characterization of many hits.

Materials

96-well polypropylene plate (Corning)

200ul, 1000ul pipette men, tips
Parafilm

Dissecting microscope

HPLC/MS grade Acetonitrile

HPLC/MS grade H2O

5mg/ml α-Cyano-4-hydroxycinnamic acid in 1:1 Acetonitrile:H2O

DMSO

384-well polypropylene plate (i.e. Whatman Microplate Devices Uniplate, 384 wells, 80uL, Polypropylene, V bottom, cat # 7701-5101)

Sticky covers for 384-well plate (i.e. Corning Incorporated costar cat # 6570)

Access to a Tandem TOF/TOF Mass Spectrometer (we have an Applied Biosciences 4700 Proteomics Analyzer) and MSMS plate and someone who can help you use it if you’re not properly trained, as well as cleaning reagent, soft bristled toothbrush, polish, kimwipes, and methanol for cleaning the MSMS plate

Access to a Microarray spotter (we use a TeleChem International NanoPrint LM 360 with MP946 Micro Spotting Pins) and someone who can help you use it if you’re not properly trained

Ethanol

Procedure

1. While visualizing all hits beads at the bottom of the eppendorf tube, drain most of the water using a 1000ul pipetteman.
2. Add 1ml HPLC grade Acetonitrile.
3. Under the dissecting microscope, visualize hit beads in your eppendorf tube. Let them settle to the bottom of the tube and transfer some of them with a 200ul pipetteman to an empty well of a 96-well plate.
4. With the 200ul pipetteman, pipette one bead at a time to adjacent wells. If too many beads are ejected into a well, try to suck all but one back into the tip and transfer extra beads to the next well. This takes some practice but several beads per minute can be transferred with proper technique.
5. Repeat steps 3-4 until all hit beads are transferred to 96-well plate(s).
6. Let the acetonitrile evaporate (most wells should be evaporated by the time you get to the last well).
7. Weigh out 30mg (20-30mg) CNBr into an empty eppendorf tube. Add 500ul HPLC grade acetonitrile, 400ul glacial acetic acid, 100ul HPLC grade water.
8. Add 20ul of CNBr solution to each well containing a bead (plus one or more empty wells for negative control).
9. Cover with sticky cover and parafilm and place on shaker overnight at room temperature.
10. Next morning, take off the cover and leave in hood for a few hours until dry.
11. Add 20ul HPLC grade water to each well, cover with sticky cover and leave on shaker 1hr.
12. Add 10ul of DMSO to one well per hit bead to 384-well plate
13. Transfer 10μl of solution from each of the 96-well plate wells containing cleaved hits to the 384-well plate. Make sure tubes are marked properly and the location of each compound in the 384-well plate are logged properly in your lab book. Cover the plate with a sticky cover or proceed to spotting onto microarray slides.

14. Add 10μl of acetonitrile to each well of the 96-well plate.

15. Cut a piece of parafilm, fold creases in it with the inside of the creases facing the paper cover. Remove the paper cover.

16. Add 1μl from tube 1 to the first crease in the parafilm.

17. Add 1μl of 5mg/ml α-Cyano-4-hydroxycinnamic acid in 1:1 Acetonitrile:H2O to the 1μl on the parafilm, pipette up and down to mix, and spot ~1μl onto a spot on the MSMS plate.

18. Repeat steps 10-11 for all of the wells of the 96-well plate containing hit compounds (and negative wells). Make sure to record which wells correspond to which spot on the MSMS plate.

19. Sequence hits.