SOP #K-002 Millipore metabolic rat Milliplex assay

Assay: Millipore Metabolic Rat, 10 Analytes

Assay detail: 10 beads

Format: Half 384 well-plate assay is an effective format that manages the maximum number of samples economically, and allows collection of sufficient samples to half fill the plate. The second half will be performed **within 1 month** of first half plate, each half plate accommodates 12 rats at 5 samples per rat and duplicate controls (24 x 4 wells)

Note: Because these kits are designed for 96 well plates, for every four kits used, order one extra set of assay buffer, detection antibody and Streptavidin-PE since these are limiting when we create equivalent of 4 assays from one 96 well kit.

Important Steps

1. Remove the kits from the cold room and allow everything to come to room temperature, about 30min when starting the assay.
2. Switch on the Mini-Orbital Shaker (Bellco Biotechnology, Vineland, New Jersey, USA) to warm up the bearings, set it to 6. Shut it off any time after 15min.
3. Create labels on the DYMO LabelWriter 450 Turbo printer using specific labels (Labtags Company, Cat#ED1F-079) for C24 Standard and serum matrix. The label template is named *Millipore Meta Rat Standards and matrix tubes*. Change the expiration dates to one month from the current date. If the label writer is not available, use colored round stickers that can be used to create the labels.
4. Get all the reservoirs you will need for the day and place them upside down on the bench (this prevents them from room dust). (Usually 4 are required: Assay Buffer, Serum Matrix, Bead, Bead diluents)
5. Organize the samples in a frozen PCR tube freezer rack (IsoFreeze, product #16361) the way you will load them on your plate.
6. Prepare the 384 filter plate. **Tape the plate to an inverted lid to protect the filter bottom. Do this for the duration of the assay.** Cover half the plate with a plate sealer if you are only using half the plate). (Cut plate sealer in half).
7. Remove the metal cap protectors from the **Serum Matrix, Standard and Bead diluents**, tubes provided in the Kits using forceps.

8. Reconstitute the **Serum Matrix** by adding 1ml of sterile Millipore water.

9. Reconstitute the **Standard** by adding **167µl** of sterile **Millipore water (Millipore A10)** . Set timer for 10min. This is the minimum time for reconstitution before you can make the standard curve.

10. Take the (orange) PCR tube rack (see photo) (21 x 8 tube holders) and place the pre labeled empty tubes in the rack. The highest number on the left.

    in row A **24/22/20/18/16/14/12/10/8/6/4/2/0**

    in row F **23/21/19/17/15/13/11/9/7/5/3/1/0**

SOP# Making the STANDARDS for the Millipore metabolic rat milliplex assay

1. Take the (orange) PCR tube rack and place the pre-labeled empty tubes in the rack. The highest number should be on the left.

   24/22/20/18/16/14/12/10/8/6/4/2/0

   23/21/19/17/15/13/11/9/7/5/3/1/0

2. Open the **Assay buffer** and pour about half into the reservoir labeled with assay buffer (note: 30 ml is insufficient volume for two 384 half plates)

3. Using the p125 pipettor (yellow), add 25µl of Assay buffer to all tubes **EXEPT for tube 24 and 23**

4. **Prepare the pipette: Preset>stepper>pre-step, 25µl, 2x, 9, 7 (**it will fill 50µl and you can pipette twice)

5. Add 15ul of assay buffer in tube 23 using a normal pipette.

6. Spin all of the tubes in the platefuge (PlateFuge, Benchmark Inc) to make sure all of the buffer is at the bottom with **no bubbles,** ~1-2min. **Important the tubes must be bubble free.**

7. Mix the standard vial well from all sides. Centrifuge the standard vial in the platefuge.

   Using the P200 pipette transfer the standard to the **C24** tube. Transfer the maximum volume possible.

8. Add 30µl of tube C24 (standard) into tube C23. Make sure you mix C24 with the pipette prior to extracting the 30ul and mix 4x with the pipette when dispensing into tube C23.

9. Make sure that the C24-C0 is in row A and the C23-0 is in row F (in the orange PCR rack)

10. Get out the special pipette box for **standards dilution (see photo)** with the right spacing marked by the black lines and put 12 tips in each of the 2 rows.

11. On the 125 Eclip-tip pipette (Yellow) (E1-Clip, Thermo Scientific) go to programs and load "**Dilute Stds**"

Pick up 2 tips, Starting at the first tube of each row (24,23)

a. Put the tips to the bottom of the first tube
b. Trigger, mix
c. Trigger mix
d. Beep
e. Touch off on sides
f. Move to second tube bottom
g. Trigger mix
h. Trigger mix
i. Pull up bit above liquid with tip touching side
j. Trigger to dispense (25µl)
k. Tip over bucket
l. Trigger
m. Trigger eject
n. Close second tube
o. Open third tube
p. Repeat for the rest of the tubes **except C0s**, you will have 1 tip left in each row.

Remove **C4** from the evens and **C0** from the odds, weas these will not be used

Flip the order of the even tubes

C0/C2/C6/C8/C10/C12/C14/C16/C18/C20/C22/C24

C23/C21/C19/C17/C15/C13/C11/C9/C7/C5/C3/C1

See separate sheet for the dilution

SOP# Setting up the BEADS for the Millipore metabolic rat milliplex assay

The following are required:

- Teal clip tips
- 5x16 Eppendorf rack
- Multi-vortex tube holder
- Vortex
- Large bucket (useful when dealing with wet tubes when vortexing)
- Sonicator (use distilled H_20) (in our lab use gallon jar with green label)
- Tray labeled for mixed beads
- Tray labeled for diluents

The beads are made for two half plates

The beads are good for 1 month

Before making the beads the filter plate must be pre-wet for at least 10min

Add 75µl of **Assay Buffer** to each well using the teal (15-1250µl) Eclip tip use only 6 pipette tips. The spacing should be set to narrowest and you will be adding to every other row. Do some mini drops on the plate to get the liquids to the bottom of the wells.

Use only 6 tips (pipette twice in the same row first in tube 1 and then in tube 2)

Row

#	#	#	#	#	#	#	#	#	#	#	#
1		1		1		1		1		1	
	2		2		2		2		2		2

Preset>stepper>pre-step, 75ul, 12x, 3, 4 (will pull up 900µl)

After adding the assay buffer replace plate lid and place the plate on the blue shaker and tape it securely and shake the plate for at least 10min. (Longer is ok).

1. Take each bead vial out and verify the bead identity, pay attention to active vs total forms of analytes.
2. Unscrew the lid a little and screw it back on but not super tight.
3. Place each vial into the multi tube vortex holder.
4. Place the vortexer in the large bucket (to prevent splashing of water on outside of vials).
5. Label a blue screw top 5ml tube with mixed beads and date, and wrap it in aluminum foil (to **protect it from light**).
6. Take tray labeled with "**bead diluents 384 well half plate**" and "**mixed beads tray**" they have to be cleaned and dust free.
7. Fill the bead diluents tray with the whole vial of bead diluents.
8. **Use Clip-tip 15-1250 µl teal electronic pipettor**
    - Use program forward:
    - Set volume = 100
    - Speed up = 5
    - Speed out = 2
9. Tip box 4 sets of 5 tips
    - * * * * *
    - * * * * *
    - * * * * *
    - * * * * *
10. **Check Branson Sonicator**

    The water must be at the water line level. If not add distilled H_20. (see photo)
11. Place all 10 tubes in the multi-tube vortex holder. Put lid on. Sonicate the tubes for 30sec.

    Don't get the caps wet!
12. Vortex the tubes for 1min. Work quickly!!
13. Take the bead vials out; put them in the Eppendorf rack 5 on one side, 5 on the other
14. Open all the lids (put them next to the vial).

Set the pipette to the widest setting 12.9

15. Take up 100µl from the first 5 tubes (each tube contains 200µl beads)

16. Dispense into the mixed bead tray.

DO NOT eject tips.

17. Take second 100µl from the 5 tubes and dispense into the mixed bead tray.

18. Take 100µl bead diluents with new tips and rinse the tube with the 100µl bead diluents and dispense into mixed bead tray.

19. Rinse the tubes for the second time with 100µl bead diluents and dispense into the mixed bead tray.

20. Repeat with the second 5 tubes.

21. Use a 1ml pipette mix up the beads very well and transfer to the 5ml tube with aluminum foil around.

SOP# for loading the half 384 milli-metabolic-rat_ plate

1. Get samples ready

If thawed vortex them and spin them down. Put them back on ice.

2. Get standard tubes ready

Open lids and put rack cover over the rack.

3. Get beads ready

Vortex mixed beads (check pellet) sonicate for 10-15sec.

4 Get the standards ready

From the PCR tube line used for standards (see photo)

Remove (cut out) the No “4” dilution tube as it is not used (on the even dilutions)

Remove (cut out) the No “0” dilution tube as it is not used (on the odd dilutions)

Flip evens (so that they go from 0 to24) (left to right) (do not flip odds)

0 2 6 8 10 12 14 16 18 20 22 24

23 21 19 17 15 13 11 9 7 5 3 1

5. Fill the trays with the solutions (Matrix and Assay Buffer)

6. Get the plate ready

Turn on vacuum (Model #WP6111560 Vacuum Pump, Billerica MA, 01821) (should read about -20Hg (see photo)).

Before placing the filter plate onto the vacuum manifold, remove metal support. (see photo). Wet the blue silicon rim of the manifold with water.

Remove the bottom cover (a plate lid) protecting plate. Vacuum liquid out of plate.

Tap plate on stack of paper towels, until towels showing no wet spots. Tape the protecting plate back on.

Matrix

Take a 125 Ecliptip (load always 12 tips)

Fill the matrix tray with the matrix

Use program half 384-ZT matrix **(There is a pre Step)**

Load in row AB and OP Cover row A and B after you loaded the matrix.

Assay

Fill the assay buffer into the assay tray.

Use program half 384-ZT assay **(There is a pre Step)**

Load assay buffer in row C to N

Standards

Load standards (no pre step)

Load Standards

Use program half 384-ZT Std.

Load 0 - 24 in row A and O

Load 23 - 1 in row B and P

Load Samples

Use program half 384 samples.

You are using only 5 tips at the time!

Load samples in row C-N

Row C load first half of the sample in 1 -5 (7.5µl) a1/a2/a3/a4/a5

Row C load second half of the samples in 6-10 (7.5µl) b1/b2/b3/b4/b5

See plate layout!

Loading Beads

Use program half 384ZT beads **(There is a pre Step)**

Load beads in all rows A – P

Work quickly

Be sure no liquid is hanging on the edges. If so gently drop the plate (mini drops) on the table.

Put lid back and tape it back on plate!

Prepare Bioshake (Q-Instruments, Germany)

Qcom, 2400 rpm for 25µl

1600 rpm for 75µl

- Tape plate with protection lid on the shaker.
- Don't tape over prongs.
- Don't tape over black.
- Tape it over the top too
- Tape it well on the shaker.
- Put aluminum foil over the plate.
- Put the shaker in the cold room

Lock the card wheels!!!!!!!!!!!!!!

- Plug shaker in
- Turn on computer
- Start shaker at 2400rpm
- Incubate for 16-18 h
- Turn of monitor of the computer

Clean up

1. Return mixed beads to kit (refrigerate).

   Extract as many beads as possible out of the reservoir (mix and rinsing with pipette).

2 Put leftover serum matrix in a tube and label + date.

3. Standard leftover C23 and C24 label with date put them in the box in the bottom of the -20C for Millipore-metabolic rat kit supplies

4. Pour assay buffer back

5. Check wash buffer

6. **Make wash buffer**

   540ml H_20

   60ml 10x wash buffer

7. Wash the reservoirs and let them dry.
8. Make notes regarding the plate.

Second Day

1. After 16-18 hours
2. Take out the kit and the last kit just in case. Should be room temperature before use.
3. Take out the wash buffer from the fridge. Should be room temperature before use!!
4. Pour 400 -500 ml into the short plastic bottle for the multidrop
5. Get the shaker from the cold room
   Turn the monitor back on
   Stop the shaker
   Shut down the computer
6. Bring the shaker back to the lab
   Turn on the computer and start Bioshake software
   Put the brakes on
7. Get the multidrop ready (it has 2 turn on buttons and be sure the primer container is in right position and the cover is closed right over the tubing)
   Check NO NOZZLES ARE CLOGGED
   SET SELECTOR FOR 384
   SET COLUMS FOR 12
   SET VOLUME FOR 75UL
8. Get reservoir out for detection AB and detection AB bottle
9. Yellow Ecliptip set it:

Preset>stepper>**pre-step**, 15.6µl, 8x, 9, 7

10. Turn vacuum on (take out metal support, wet silicone)
11. Vacuum liquid out of the plate.
12. Blot button of the plate on paper towels several times, until towel not longer wet
13. Get the plate on the multidrop
14. Add wash buffer, push start button
15. Vacuum liquid out, blot on paper
16. Wash 4x (until you added buffer 4x)
17. Before last vacuum pour **detection AB** in the large reservoir
18. Get yellow pipette

    Add 15.6ul **detection AB** to all wells **(there is a pre-step)**

    Preset>stepper>**pre-step**, 15.6, 8x,9 ,7
19. Put lid on (tape it on well with tape)
20. Shake for 30 min

    Cover with aluminum foil

    Shake at 2400rpm
21. Add **Streptavidin-PE** to the wells.

    (pour **Steptavidin-PE** in reservoir. 3.5ml for the half plate)
22. Use yellow pipette

    Preset>stepper> **Pre-step>** 15.6ul 8x, 9, 7 **(there is a pre-step)**
23. Stop shaker and add **Streptavidin-PE**.
24. Cover with lid and cover with aluminum foil for 30min.
25. **Turn on the Attune Cytometer**

    Start up

    Run performance test

    Load up the template named:

    Mili-metabolic-rat-plate-384_well_half
26. After 30 min shaking turn off the shaker and **change speed to 1600rpm**
27. Vacuum of the contents of the plate

28. Add 75µl wash buffer
29. Wash 3x (means fill 4x)
30. Blot plate on paper towel
31. Wash 2x more
32. Blot very well after last vacuum (no wet spots on the towel)
33. Add 75µl of wash buffer
34. Shake plate for 10 min.
35. Get a full box of tips
36. Get a 384 v bottom plate and label it with plate number and cover second half of the plate with plate sealer.
37. Get yellow Ecliptip

    Program> Transfer to 384

    This will mix and pull out 75µl of the probe.
38. **Change volume on the multi drop to 25µl.**
39. Fill the filter plate with 25µl wash buffer.
40. Reattach filter plate to the shaker. Shake for 3min.
41. Start the time shake 5sec.
42. Transfer the 25µl probe to the 384 v bottom plate
43. For repeat, hit 8 times tab and click on 5sec
44. Repeat until plate is done
45. Put plate in attune

Clean up

1. Put filter plate back to the multi drop.
2. Change volume to 75µl.
3. Add 75µl of wash buffer to the plate.
4. Put plate back to inverted plate.
5. Put cover back on and tape it to the plate.
6. Seal the plate with parafilm.
7. Wrap the plate in aluminum foil.

8. Label plate with plate number and date.
9. Put the plate in fridge.

- Put the Millipore metabolic rat kit back in the cold room.
- Wash the reservoirs.
- Clean the multi drop machine with H_20. No water drops should be in the tubes anymore.
- Push the black guard backwards.
- Take the tubes out of the bracket.

Note at SOP check set up multi drop Machine

Put the tubes in the bracket and close the black guard