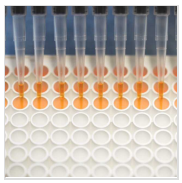

Aug 30, 2022

SPARC - Millipore Metabolic Rat Multiplex Bead Assay for Flow Cytometry

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dx.doi.org/10.17504/protocols.io.14egn82ymg5d/v1

J Paul Robinson

ABSTRACT

This protocol describes methods of performing a Milliplex Assay in half of a 384-well plate, with each half-plate accommodating 12 rats (at 5 samples per rat) and duplicate controls (24 x 4 wells).

Assay: Millipore Metabolic Rat, 10 Analytes

Assay detail: 10 beads

DOI

dx.doi.org/10.17504/protocols.io.14egn82ymg5d/v1

PROTOCOL CITATION

Doris Kemler 2022. SPARC - Millipore Metabolic Rat Multiplex Bead Assay for Flow Cytometry. **protocols.io** https://protocols.io/view/sparc-millipore-metabolic-rat-multiplex-bead-assay-baetiben

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OWNERSHIP HISTORY

Dec 11, 2019 Liz Brydon Protocols.io

Dec 13, 2019 J Paul Robinson

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30899

GUIDELINES

Using half of a 384 well-plate for the assay is an effective format that manages the maximum number of samples economically, and allows collection of sufficient samples to fill half of the plate. The second half will be performed **within 1 month** of filling the first half of the plate.

MATERIALS TEXT

Final millimetabolic assay materials (Doris Kemler) original (1).xlsx

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for safety and environmental hazards.

BEFORE STARTING

Because the Milliplex Assay 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.

Follow the Setting up the BEADS for the Millipore Metabolic Rat Milliplex Assay protocol to prepare the beads, within a month of the assay.

Preparation

1 Remove the kits from the cold room and allow everything to come to **Room temperature** (about 30 min before starting the assay).

2 Switch on the Mini-Orbital Shaker (pictured below is: Bellco Biotechnology 7744-08096 0-1150 RPM Mini-Orbital Mixer Stirrer Shaker, but newer alternatives such as the Bioshake shaker from www.Qinstruments.com are also recommended) to warm up the bearings, set it to 6. Shut it off any time after 15 min.

3 Create labels on the DYMO Label Writer 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.

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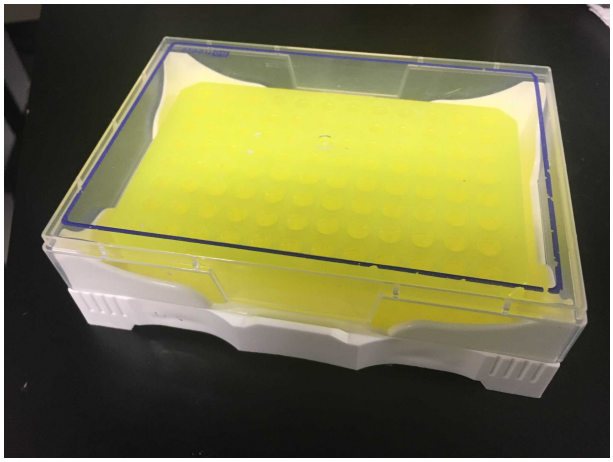
4 Get all the reservoirs you will need for the day and place them upside down on the bench (this protects them from room dust).

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. (This is done to protect the bottom of the filter plate which is delicate.) 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

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Reconstitute the Serum Matrix by adding **1 mL sterile Millipore water** (Millipore A10).

9

Reconstitute the Standard by adding **167 µL sterile Millipore water** (Millipore A10).

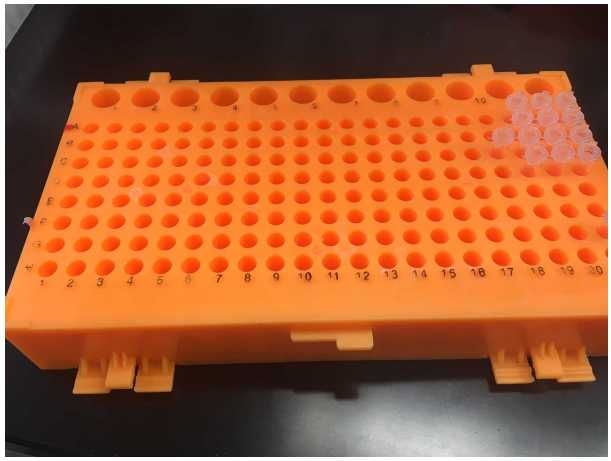
10

Set timer for **00:10:00** . This is the minimum time for reconstitution before you can make the standard curve.

11 Place the pre-labeled empty tubes in a PCR tube rack (21 x 8 tube holders; orange rack), organizing tubes with the highest number placed on the top left as such:

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



This is a convenient rack we use for these assays

Making Standards

12 Open the Assay buffer and pour about half of the bottle into the reservoir (pre-labeled with "assay buffer").

30mL is insufficient volume for two 384-well plates.

13

Using a p125 pipettor, add **25 µL assay buffer** to all tubes **EXCEPT for tube 24 and 23**.

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14 Prepare the pipette: Preset->Stepper->pre-step, 25µl, 2x, 9, 7.

It will fill 50µl and you can pipette twice.

15

Add **15 µL assay buffer** in tube 23 using a normal pipette.

16

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 present**, roughly **00:01:00** (up to 2 minutes is fine).

The tubes must be bubble free before proceeding.

17

Mix the standard vial well from all sides.

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18

Centrifuge the standard vial in the platefuge.

19

Use a P200 pipette to transfer the standard to the **C24 tube**.

> Transfer the maximum volume possible.

20

With a pipette, mix the C24 tube contents thoroughly, immediately prior to the next step.

21

Add **30 µL** of tube C24 (standard) into tube C23 and **pipette-mix four times after dispensing**.

22 Make sure that tubes C24-C0 are in row A and C23-C0 are in row F (in the orange PCR rack).

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

24 Using a 125 Eclip-tip pipette (E1-Clip, Thermo Scientific), go to programs and load “Dilute Stds” (yellow pipette)

25 Perform the following steps with the Eclip-tip pipette:

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25.1 Pick up 2 tips (from the special pipette box).

Start from the first tube of each row (i.e. C24 and C23).

25.2 

For **second** tube (C22/C21):

- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **second** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close second tube
- Open third tube
- Pick up 2 tips (from the special pipette box)

25.3 

For **third** tube (C20/C19):

- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **third** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close third tube
- Open fourth tube
- Pick up 2 tips (from the special pipette box)

25.4 

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For **fourth** tube (C18/C17):

- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **fourth** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close fourth tube
- Open fifth tube
- Pick up 2 tips (from the special pipette box)

25.5 

For **fifth** tube (C16/C15):

- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **fifth** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close fifth tube
- Open sixth tube
- Pick up 2 tips (from the special pipette box)

25.6 

For **sixth** tube (C14/C13):

- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **sixth** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket

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- Trigger
- Trigger, eject
- Close sixth tube
- Open seventh tube
- Pick up 2 tips (from the special pipette box)

25.7 

For **seventh** tube (C12/C11):
- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **seventh** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close seventh tube
- Open eighth tube
- Pick up 2 tips (from the special pipette box)

25.8 

For **eighth** tube (C10/C9):
- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **eighth** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close eighth tube
- Open ninth tube
- Pick up 2 tips (from the special pipette box)

25.9 

For **ninth** tube (C8/C7):
- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)

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- Beep
- Touch off on sides
- Move to **ninth** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close ninth tube
- Open tenth tube
- Pick up 2 tips (from the special pipette box)

25.10 

For **tenth** tube (C6/C5):
- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **tenth** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close tenth tube
- Open eleventh tube
- Pick up 2 tips (from the special pipette box)

25.11 

For **eleventh** tube (C4/C3):
- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **eleventh** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close eleventh tube
- Open twelfth tube

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- Pick up 2 tips (from the special pipette box)

25.12 

For **twelfth** tube (C2/C1):
- Put the tips to the bottom of the **first** tube
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Beep
- Touch off on sides
- Move to **twelfth** tube bottom
- Trigger, mix (1/2)
- Trigger, mix (2/2)
- Pull up bit above liquid with tip touching side
- Trigger to dispense (25µl)
- Tip over bucket
- Trigger
- Trigger, eject
- Close twelfth tube

Do not add to C0 tubes. You should have 1 tip left in each row of the tip box at this point.

26 ⚠

Remove C4 from the even number row and C0 from the odd number row (these tubes will not be used). We do not use the C4 dilution.

27 Flip the order of the even tubes so that the numbers are organized as such:
C0/C2/C6/C8/C10/C12/C14/C16/C18/C20/C22/C24
C23/C21/C19/C17/C15/C13/C11/C9/C7/C5/C3/C1

Loading the Half-filled 384-well Plate

28 Prepare samples.

If thawed, vortex and spin samples down. Keep on ice.

29 Prepare standard tubes - open lids and put rack cover over the rack.

30 Prepare beads - vortex mixed beads (check pellet) and sonicate for **00:00:10** (up to 15 seconds).

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Make sure to follow Setting up the BEADS for the Millipore Metabolic Rat Milliplex Assay prior to getting started.

31 Prepare standards.

31.1 Remove/cut C4 dilution tube from the even dilutions and the C0 from the odd dilutions (neither are used).

31.2 ⚠

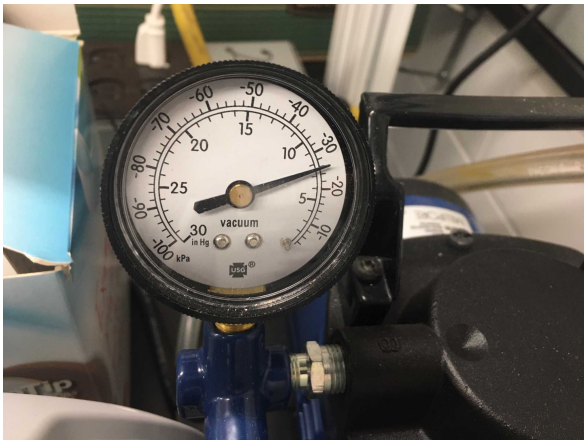
Organize the even dilution row so that they list 0-24 when reading from left to right. **Do not flip the odd dilutions.**

Plate numbers should read like:
0 2 6 8 10 12 14 16 18 20 22 24
23 21 19 17 15 13 11 9 7 5 3 1

32 Fill the trays with the Matrix and Assay Buffer.

33 Prepare plates.

33.1 Turn on vacuum (Model #WP6111560 Vacuum Pump, Billerica MA, 01821) and ensure it reads about -20Hg.



33.2 Before placing filter plate onto the vacuum manifold, remove metal support and wet the blue silicone rim of the manifold with water.

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33.3 Remove the bottom cover (plate lid) protecting the plate.

33.4 Vacuum liquid out of the plate.

33.5 Tap plate on stack of paper towels, until towels no longer show wet spots.

33.6 Tape the protecting plate back on.

34 Load Matrix.

34.1 Load 12 tips on the 125uL Ecliptip pipette.

34.2 Fill the matrix tray with matrix solution.

34.3 Select program "half 384-ZT matrix". (**There is a pre Step.)**

34.4 Load matrix in **rows AB and OP** (shaded **green** in image below). Cover row A and B immediately after loading matrix.

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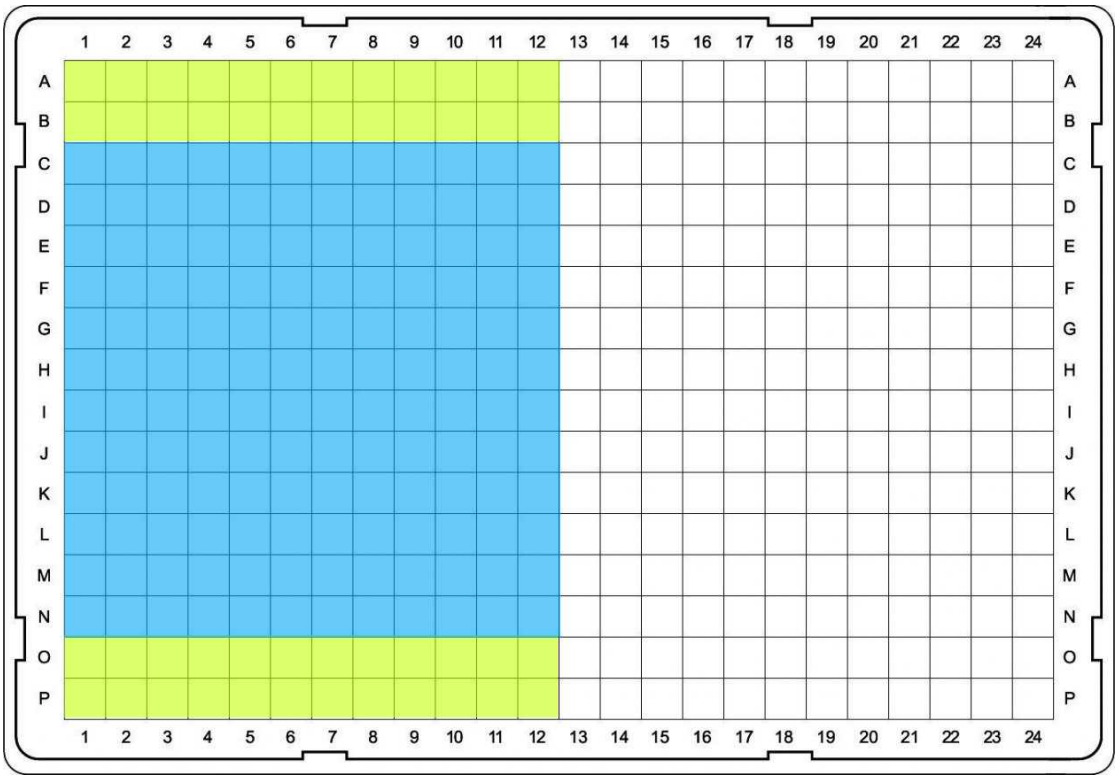

35 **Load Assay.**

35.1 Fill assay tray with assay buffer.

35.2 Select program "half 384-ZT assay". (**There is a pre Step.**)

35.3 Load assay buffer in **rows C to N** (shaded in **blue** below).

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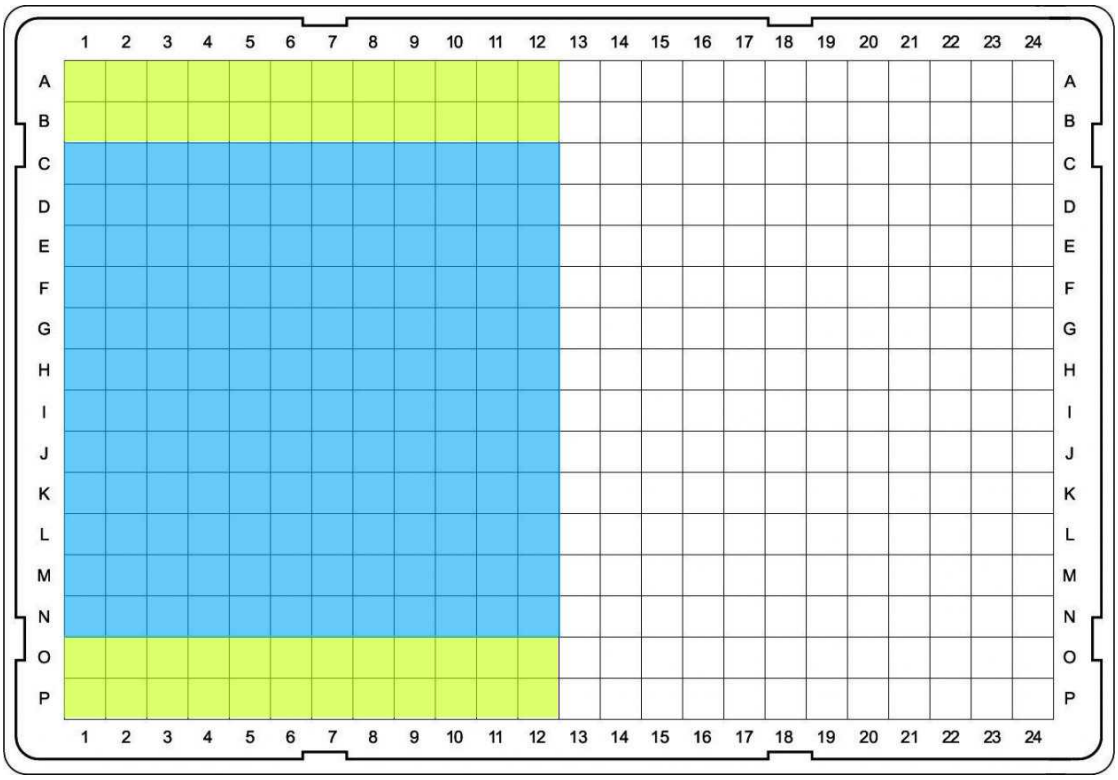

36 Load standards. (**No pre step.**)

36.1 Select program "half 384-ZT Std".

36.2 Load even row (0-24) in row A and O.
Load odd row (23-1) in row B and P.

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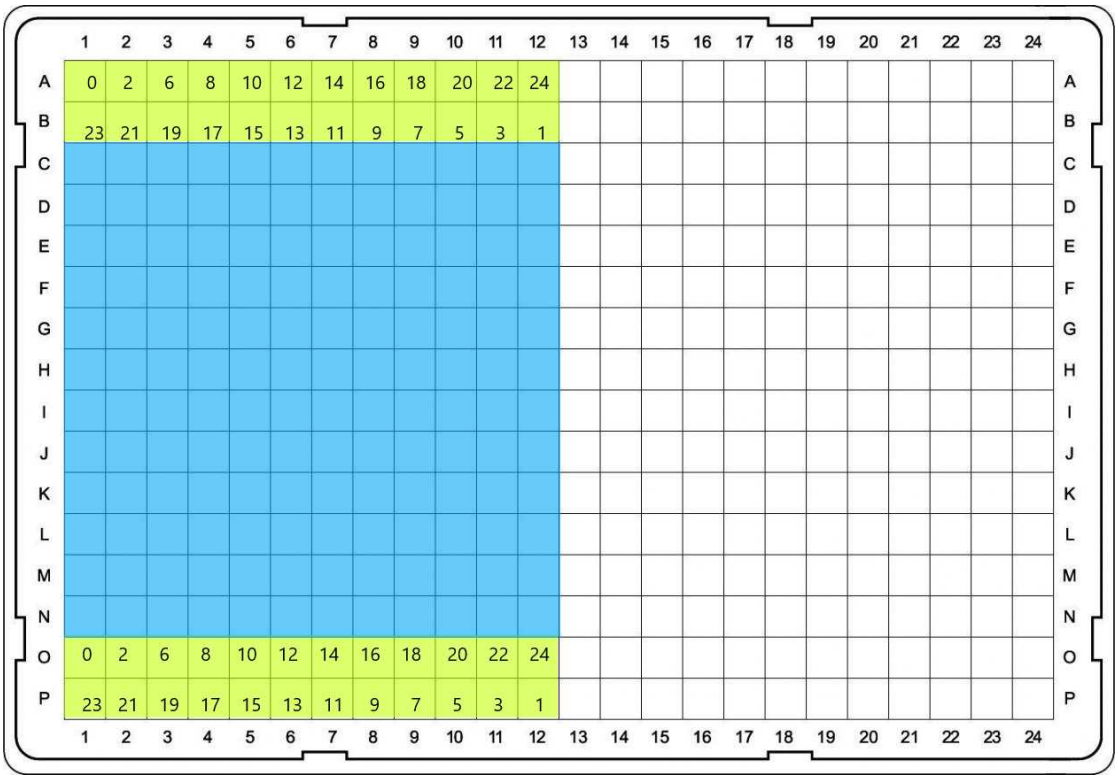

37 **Load samples.**

37.1 Select program "half 384 samples" and be sure to *use only 5 tips with the pipette at the time*.

37.2 Load samples in row C - load 7.5ul of the first half of sample in columns 1-5 and 7.5ul of the second half of sample in columns 6-10.

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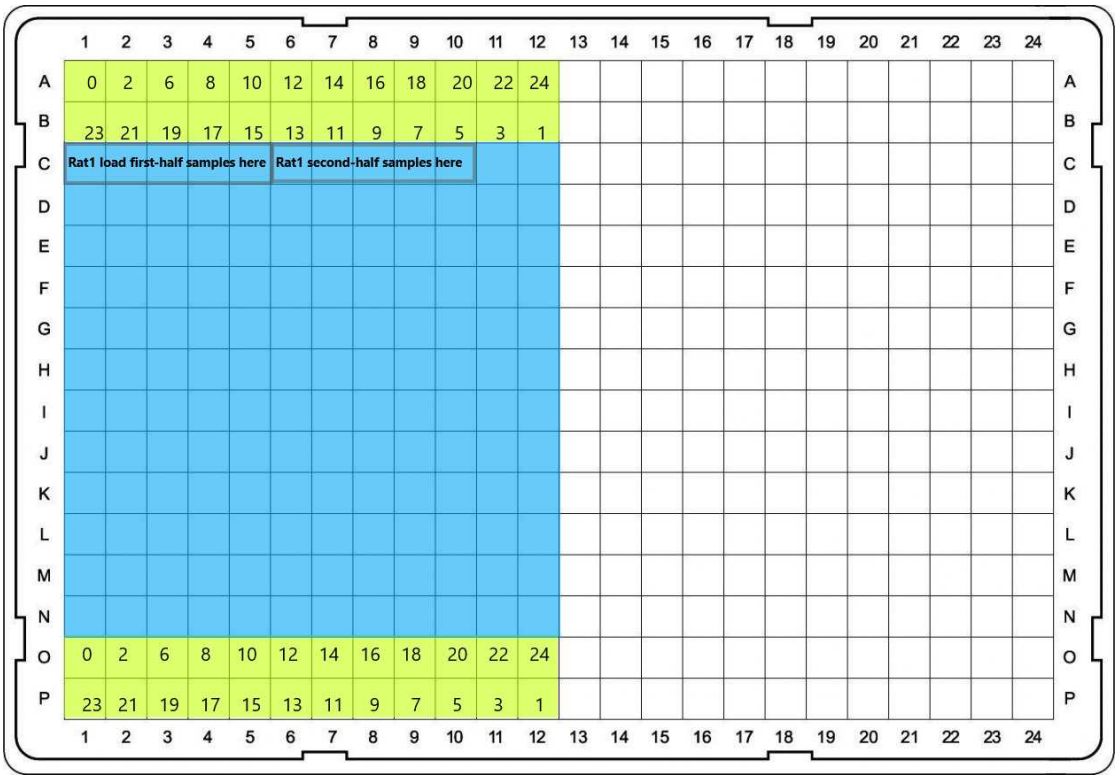
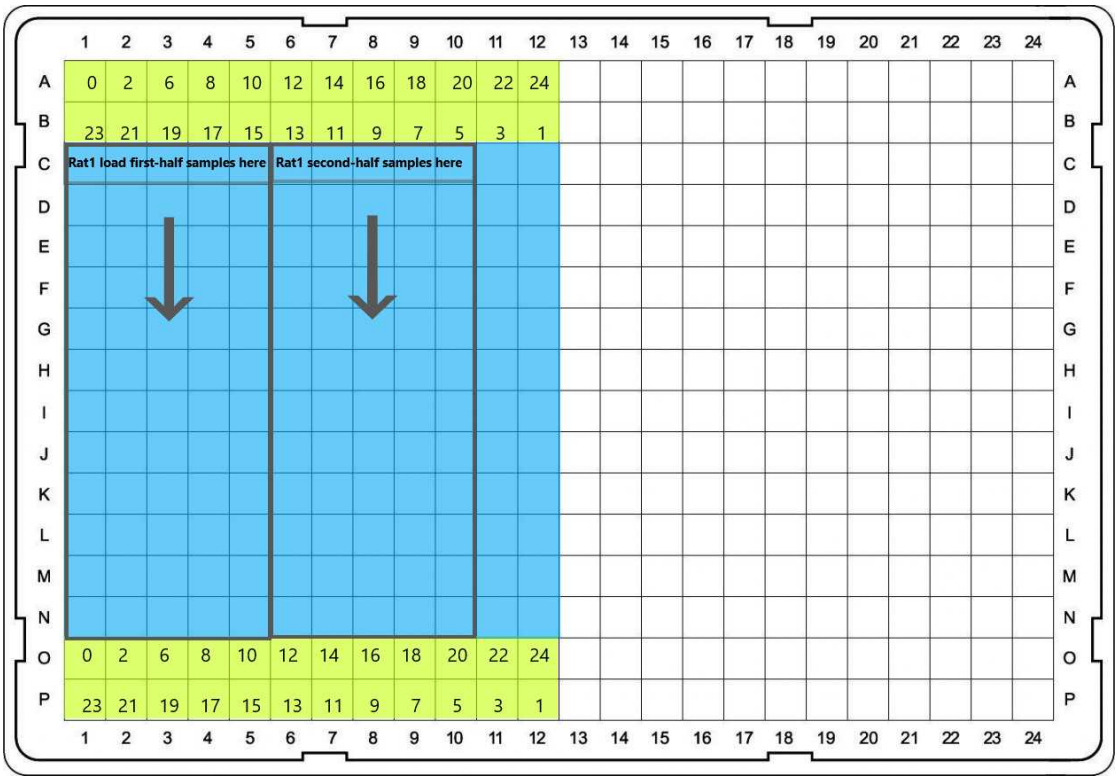


37.3 Continue loading samples in rows D-N following the column structure from the previous step (first-half samples in columns 1-5 and second-half samples in columns 6-10).



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38 Load beads.

Be sure to work quickly when working with beads.

38.1 Select program "half 384ZT beads". (**There is a pre Step.**)

38.2 Load beads in all rows A-P.

Be sure no liquid is hanging on the edges. If so, gently drop the plate (mini drops) on the table until all liquid is collected at the bottom.

38.3 Put lid back and tape it back on plate.

Prepare Bioshake

39 Bioshake: Q-Instruments, Germany.
Qcom, 2400 rpm for 25 µl
1600 rpm for 75 µl

40 Tape entire plate with protection lid on the shaker. The Bioshake is one of the best shakers available and our experience is that regular lab bench shakers are not adequate for proper dispersal of the small number of beads in each well of a 384 well plate. THIS IS A CRITICAL STEP.

Don't tape over prongs or black area.

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Tape it well, including over the top of the plate.

41 Put aluminum foil over the plate TO PROTECT FROM LIGHT.

42 Put the shaker in the cold room.

43 **Lock the card wheels IF THE SHAKER IS ON A CART.**

44 Plug shaker in and turn computer on (some Bioschake devices can be run indepenendent of a computer but some (ie the ones generally used on automation systems) require a computer.

45 Start shaker at 2400rpm.

46 

Incubate for **16:00:00** (up to 18 hours). Turn off monitor of computer.

Cleaning/Retrieving reagents

47 Return mixed beads to kit (previously in reservoir tray) and refrigerate.

Extract as many beads as possible out of the reservoir by mixing and rinsing with a pipette.

48 Put leftover serum matrix in a tube and label/date.

49 Label/date and store leftover C23 and C24 standard solutions at **-20 °C** .

50 Pour extra assay buffer back into original container.

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51 Check wash buffer.

If needed, make more: add **60 mL** 10x wash buffer to **540 mL** H_2O.

52 Wash reservoirs and leave them to dry.

53 Make notes regarding the plate.

Preparing for Detection

54 [**Second Day**, after 16-18 hours]

Warm kit (and last kit just in case) and wash buffer to **Room temperature** .

55 Pour 400-500mL of wash buffer into short plastic bottle for the multidrop.

56 Locate the shaker in the cold room.

56.1 Turn computer monitor back on and stop shaker. (only if shaker is computer operated modle, otherwise just suspend operation)

56.2 Shut down the computer.

57 Bring the shaker back to the lab and set it up.

57.1 Turn on the computer and start Bioshake software.

57.2 Put the brakes on.

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58 Prepare the multidrop. Check both power buttons are switched on, that the primer container is in the correct position and the cover is closed right over the tubing.

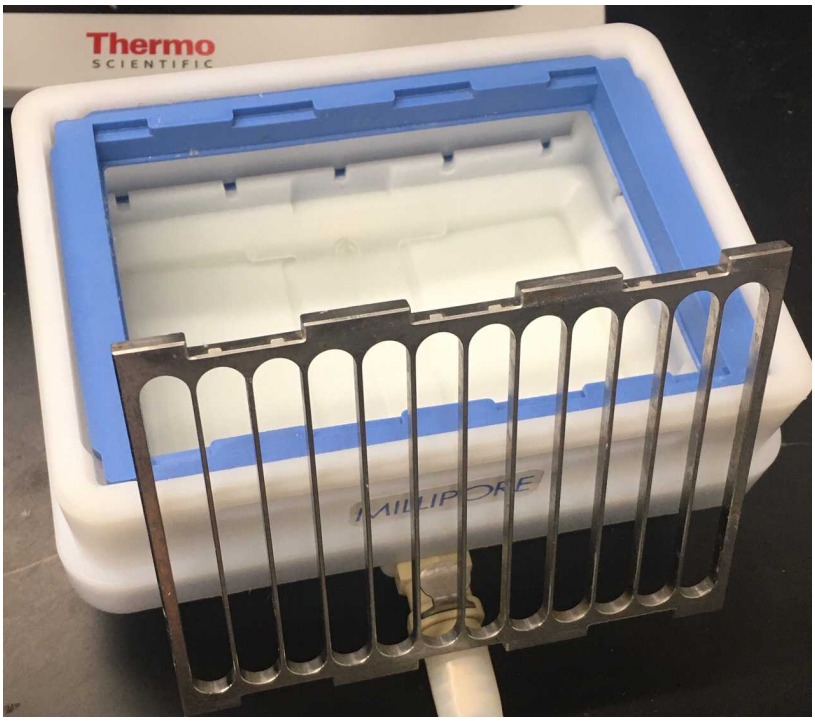
Ensure the following is correct:
- NO NOZZLES ARE CLOGGED
- SET SELECTOR FOR 384
- SET COLUMNS FOR 12
- SET VOLUME FOR 75UL

59 Get reservoir out for detection AB and detection AB bottle.

60 Set program on Ecliptip pipette to Preset>stepper>**pre-step**, 15.6µl, 8x, 9, 7.

61 Turn vacuum on and perform the following (using it at about -20Hg):

61.1 Before placing filter plate onto the vacuum manifold, remove metal support and wet the blue silicone rim of the manifold with water.



61.2 Vacuum liquid out of the plate.

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61.3 Blot button of the plate on paper towels several times, until towel is no longer wet.

61.4 Put the plate on the multidrop.

61.5 

Add wash buffer using bottle, push start button.

61.6 Vacuum liquid out, blot on paper until no longer wet. [you will repeat the wash 4x below]

61.7 

Repeat wash: add wash buffer (1/4)

61.8 Repeat wash: vacuum liquid out, blot on paper until no longer wet (1/4)

61.9 

Repeat wash: add wash buffer (2/4)

61.10 Repeat wash: vacuum liquid out, blot on paper until no longer wet (2/4)

61.11 

Repeat wash: add wash buffer (3/4)

61.12 Repeat wash: vacuum liquid out, blot on paper until no longer wet (3/4)

61.13 

Repeat wash: add wash buffer (4/4)

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61.14 Repeat wash: before last vacuum, pour **detection AB** in the large reservoir. (4/4)

61.15 Repeat wash: vacuum liquid out, blot on paper until no longer wet (4/4)

62 

Use yellow Ecliptip pipette to add **15.6 µL detection AB** to all wells.

Use program "Preset>stepper>**pre-step**, 15.6, 8x,9 ,7"

63 Put lid back on and tape it on well using tape.

64 Cover plate with aluminum foil and shake for **00:30:00** at 2400rpm.

65 Pour **3.5 mL Streptavidin-PE** in reservoir.

66 Stop shaker.

67 

Use yellow Ecliptip pipette to add **15.6 µL Streptavidin-PE** to all wells.

Use program "Preset>stepper>**pre-step**, 15.6, 8x,9 ,7"

68 Cover plate with aluminum foil and shake for **00:30:00** at 2400rpm.

Citation: Doris Kemler SPARC - Millipore Metabolic Rat Multiplex Bead Assay for Flow Cytometry
https://dx.doi.org/10.17504/protocols.io.14egn82ymg5d/v1

69 Turn on/set up the Attune Cytometer

69.1 Start up and run performance test.

69.2 Load up template named "Mili-metabolic-rat-plate-384_well_half".

70 After shaking, turn off the skaker, remove plate and **change speed to 1600rpm**.

71 Vacuum off the contents of the plate.

72

Add **75 µL wash buffer** .

73 Wash three more times:

73.1 Vacuum off the contents of the plate. (1/3)

73.2

Add **75 µL wash buffer** . (1/3)

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73.3 Vacuum off the contents of the plate. (2/3)

73.4 

Add **75 µL wash buffer** . (2/3)

73.5 Vacuum off the contents of the plate. (3/3)

73.6 

Add **75 µL wash buffer** . (3/3)

74 Vacuum off the contents of the plate. (3/3)

75 Blot on paper towel.

76 Wash twice more:

76.1 

Add **75 µL wash buffer** . (1/2)

76.2 Vacuum off the contents of the plate. (1/2)

76.3 

Add **75 µL wash buffer** . (2/2)

76.4 Vacuum off the contents of the plate. (2/2)

77 Blot plate very well, until no wet spots show up on paper towels.

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78 Add **75 µL wash buffer** .

79 Shake plate at 1600rpm for **00:10:00** .

80 While the plate is shaking, get a box full of tips and a 384 v-bottom plate. Label the plate with the plate number and cover the second half of the plate with plate sealer. (Note: the second half of the plate is not used)

81 Set the yellow Ecliptip pipette program to "Transfer to 384" (this program will mix and pull **75 µL** of probe).

82 **Change volume on the multi drop to 25 µL .**

83 Fill filter plate with **25 µL wash buffer** .

84 Reattach filter plate to the shaker.

85 Shake for **00:03:00** . Minutes

86 Start the time shake 5 seconds.

87 Transfer the **25 µL** probe to the 384 v-bottom plate.

88 For repeat, hit 8 times tab and click on "5 sec". Repeat until plate is done.

89 Put plate in Attune plate holder. (Note the Attune plate holder cannot use filterplates becuase of the calibration bar that sits below the plate. This is the reason the filter plate has to be transferred to the regular plate. If you

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use the Beckman CYtoflex however, you do not need to transfer the filter plate as it can be placed directrly onto the Cytoflex cytometer.

Cleaning Up

90 Put filter plate back to the multi drop.

91 Change volume to 75µl.

92 Add **75 µL wash buffer** to the plate.

93 Put plate back to inverted plate.

94 Put cover back on and tape it to the plate.

95 Seal the plate with parafilm.

96 Wrap the plate in aluminum foil.

97 Label plate with plate number and date.

98 Put the plate in fridge.

99 Put the Millipore metabolic rat kit back in the cold room.

100 Wash the reservoirs.

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101 Clean the multi drop machine with H_2O. No water drops should be in the tubes anymore.

101.1 Push the black guard backwards.

101.2 Take the tubes out of the bracket.

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