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Bare Transwell assay with flow cytometry count for HL-60 chemotaxis

This protocol has been used with KW HL-60 (including 5-day Amaxa-transfected cells) and pMIG HL-60 cells.
4-7 day DMSO-differentiated HL-60 neutrophil-like cells have been used successfully in this assay, but 5-6 days is best
(5-day for Amaxa-transfected cells 4-5hrs post-transfection, 6-day for non-transfected or stable cell lines)
(Also, do not use freshly thawed cells. Wait until at least cells differentiated on day of passage 5, otherwise they may not migrate well).
It has been optimized for use in a 12-well plate.

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Materials / equipment:
12-well Cell Culture Inserts (BD), 3um pore size, PET, transparent (BD Falcon #: 353181)
12-well Companion Plate (BD) (BD Falcon #: 353503)
5-6 day HL-60 neutrophil-like cells differentiated in 1.3% DMSO
Sigmacote (Sigma)
Flow Cytometry Absolute Count Standard™ (Bangs Laboratories #: 580)
Forceps (sterilize 15 min in ethanol, keep in sterile hood in UV)
Various chemoattractant stock solutions
HL-60 media

0.5 M EDTA pH 7.4 (note: have been using pH 8.0 which seems to work fine)

Cytofix Solution (<write item number and vendor!>)

Biosafety laminar flow hood
HTS Flow Cytometer (LSR II)

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In the hood:

Open the 12-well Companion Plate.

Pipet 500uL Sigmacote into the first well of the Companion Plate. Immediately pipet back up this solution (holding the entire plate at an angle towards you helps with this) and pipet it into the next well. Repeat until a thin layer of Sigmacote has been applied to every well.

(The Sigmacote should dry rapidly as it is in an organic solvent - hexane, I believe).

Add chemoattractant-containing media (triplicates) into lower chambers.
Carefully lower the inserts into the media (one edge and then the other like putting a coverslip on a slide, to minimize risk of trapping bubbles). Ideally, use forceps.

(Note that the Cell Culture Inserts have a correct orientation that allows them to seat properly and allow proper fit of the lid. The edge of the Cell Culture Insert should fit in the groove in the companion plate.)

Equilibrate for about 20 minutes.
(Comment by Jason: Does equilibrating in this way decrease the amount of time that the gradient is stable / steep? This has not been thoroughly tested, though the current protocol does work well.)

Count cells with hemocytometer and spin them down in a 15mL centrifuge tube (1000rpm x 5min).
Spin down enough cells to have at least 100,000 cells per well (plus a little extra for wiggle room).

[
DiD staining of WT cells (to have WT cells in same well as transiently transfected population for internal control):
In 15mL BD conical:
Spin down / resuspend cells @ 1e6/mL in RPMI 1640 base medium (without FBS, A/A)
Add 5 uL DiD, invert to mix.
Incubate for 15-20 minutes @ 37 C (loosen cap).
Spin down.
Put fresh medium + FBS
]

While spinning down cells, make dilutions of chemoattractant solutions in HL-60 media.
Make 1.2 mL of solution for each well.

Aspirate supernatant from centrifuge tube and dilute cells to 250,000 cells/mL in HL-60 media. Resuspend using a P-1000 tip.
(If desired, you can count the cells again at this point to confirm cell concentration)
(Note: in case of "checkerboard" controls or chemoattractant competition assays, etc, you may wish to dilute cells for some wells into a solution with chemoattractant. In these cases you may want to resuspend these cells in solution other than HL-60 media.)
[
If doing transiently transfected cells (co-transfect GFP) with internal control of WT cells (DiD-stained),
have 125,000 cells/mL transfected and 125,000 cells/mL untransfected for total 250,000 cells/mL.
]

Pipet 1.2 mL chemoattractant solutions into Companion Plate wells.
(You can push the Cell Culture Inserts to the side of each well using the pipet tip

to faciliate pipeting of chemoattractant solutions into bottom well without removing Cell Culture Inserts. Leaving the Cell Culture Inserts in the wells seems to make it easier to avoid getting bubbles under the Cell Culture Inserts).

Briefly mix / resuspend cell solution using P-1000 and pipet 400 uL of cell solution into each Cell Culture Insert.

(400 uL contains 100,000 cells).

[

If doing transiently transfected cells (co-transfect GFP) with internal control of WT cells (DiD-stained),

use 50,000 cells of each type.

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Incubate (37C, 5% CO2, ? humidity, etc) for ~30 minutes. (We used to run this for 2 hours - this works too, but 30 min seems to be best for optimizing fold-difference in migration between stimulated and control wells).

(Note: May need more time for primary neutrophils, neutrophils extracted by peritoneal lavage, etc).

Add ~15 uL 0.5 M EDTA to bottom well. Replace lid and let sit for ~15 minutes @ 4 C.

Tap entire plate up and down to dislodge any cells loosely adherent to Insert membrane or well plastic.

(Be careful not to let any of the liquid from the top wells splash down into the bottom wells).

Use forceps to remove Cell Culture Inserts.

(You can keep the Cell Culture Inserts to remove the membrane and do other analysis like fixing / staining or prepping for SEM if desired).

Pipet up and down to mix and then transfer entire volume (~1.2mL) from bottom well into labeled 1.5mL centrifuge tubes.

Again, tilting the entire plate towards yourself helps in this step.

Note that the wells of the plates are not entirely flat - they are a little bit deeper in the center of the wells and the cells like to settle there. That is why it is helpful to pipet up and down and transfer the entire volume into an Eppendorf tube for mixing before analysis by flow cytometry.

Prepare "input cells" sample:

1.2 mL medium

400 uL of cell suspension that was put into the tops of the Transwell inserts

Prepare samples:

25 uL counting beads (Absolute Count Standard)

100 uL cell suspension from previous step (including "Input cells" sample)

100 uL Cyto-Fix solution

Run flow cytometry to count.

(Fluorescent plate reader with Calcein AM is another possible way to do counting, but we haven't tried this out in the lab yet).

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Flow cytometry:

Guava -
Use instrument settings file.
<Insert more info here?>

LSR2 -
Use Instrument settings:
(or make yourself a new experiment from Experiment template "pMig HL60 count Oct 2008". This will also give you the worksheet which has gates on it.)

FSC: 300
SSC: 220
PacBlue: 315
FITC: 350
(Note: these are likely to vary from machine to machine)

HTS Sample settings:
1.0uL/sec flow rate
75uL sample
100uL mixing vol
200uL mixing speed
2 mixes
200uL wash vol

Std curve -
(Note: You do not have to repeat this every time. But it's a good idea to do it at least once to make sure things look good.)
Follow instructions below for samples but use standard dilutions of cells (e.g. ranging from 1,000/mL to 2.5e5/mL).

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Samples -

Vortex / mix / shake Flow Cytometry Absolute Count Standard.

Pipet 25 uL of Absolute Count Standard into each sample well of flow cytometer HTS sample plate.

Pipet cell suspension up and down to mix. Pipet 225 uL into each sample well of flow

cytometer HTS sample plate.

For stopping gate, choose Absolute Count Standard gate -> 5,000 events.

Or just record 10,000 total events or so if you prefer.

Run samples.

(Note: Bottle of Absolute Count Standard gives count / mL for the bottle -> approximately 1e6/mL).

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Analysis:

Gating the cell population and the Absolute Count Standard population using a scattergram with SSC-A on the y-axis and Pacific Blue-A on the x-axis works well, provided that the voltages are set correctly so as to separate the two populations optimally.

Given that the concentration of Absolute Count Standard in the sample is known, you can determine the concentration of cells in the sample by the ratio of standard to cells and back-calculate the # of cells migrated, the % cell migration, and the migration index (which is the % migration of a given sample divided by the % migration of some negative control sample e.g. no chemoattractant).