AML Protocol

Obtain a spent AML or Acute Leukemia sample (either bone marrow or peripheral blood) from a clinical laboratory. If a count has already been performed on the sample by the lab, use that number to gauge how much volume of sample per tube will be needed. A concentration of 1-2 million leukocytes per tube of 100µL is common. Often, clinical samples have low volume, especially bone marrow aspirates; this may require compromising and removing some planned stimulants and time points from the experiment. To verify the concentration of cells, or if the clinical lab has not yet counted the sample, set up a 'count tube' as per the following protocol:

1. Add 10µL of sample to a sterile 12x75 5mL polypropylene tube (cat# 352053 BD)
2. Add 2µL of CD34 PE ('Pool' clone cat# IM1459U Beckman Coulter)
3. Add 2µL of CD45 APC-Alexa750 (J.33 clone cat# A71119 Beckman Coulter)
4. Mix the sample with the antibodies completely and incubate for 10 minutes at 25°C in the dark
5. Make a 1x Ammonium Chloride lysis solution (see Supplement 1a)
6. Add 400μL 1x NH_4Cl lysis solution to the sample and incubate at 25°C for 2 minutes
7. Add 10μL of AccuCount Fluorescent beads (cat# ACFP-50-5 Spherotech)
8. Refer to Supplement 2 for the method to estimate cell concentration
9. Determine the appropriate number of cells per stimulation tube (see Supplement 3)

The next step is staining the sample with surface markers to determine the phenotype. Often, this step is required before the stimulation experiment can proceed unless a comprehensive surface panel has already been completed. The goal of these tubes is to provide the investigator with an understanding of the heterogeneous markers of the AML, potentially enabling the separation of the malignant cells into separate phenotypic populations.

10. Add 1.5 million leukocytes to each of two sterile 12x75 5mL polypropylene tubes (cat# 352053 BD) labeled AML1 and AML2
11. Add 4mL of 1x NH_4Cl lysis solution to each sample
12. Incubate the samples at 25°C for 7 minutes
13. Centrifuge at 200g for 7 minutes, resuspend in 2mL wash buffer, and repeat the spin

14. Aspirate the supernatant and add 50μL of staining buffer to each (see Supplement 4)

15. Prepare the following surface panels (volume is per test):

AML 1	**AML 2**
2µL CD11b PacificBlue (cat# 558123 BD)	2µL CD45 PacificBlue (cat# MHCD4528 Invitrogen)
5µL CD15 PacificOrange (cat# MHCD1530 Invitrogen)	5µL CD34 FITC (cat# IM1870 Beckman Coulter)
5µL CD34 FITC (cat# IM1870 Beckman Coulter)	4µL CD33 PE (cat# IM1179 Beckman Coulter)
1µL CD14 PE (cat# 6603262 Beckman Coulter)	2µL CD117 PE-Cy5.5 (cat# A66333 Beckman Coulter)
2µL CD117 PE-Cy5.5 (cat# A66333 Beckman Coulter)	3µL CD13 APC (cat# 557454 BD)
2µL CD64 AlexaFluor 647 (cat# 305012 BioLegend)	Lymphoctye Markers – Dump Channel
1µL CD45 AlexaFluor 700 (cat# 560566 BD)	3µL CD10 ECD (cat# IM3608U Beckman Coulter)
Lympocyte Markers – Dump Channel	3µL CD19 ECD (cat# IM2708 Beckman Coulter)
3µL CD3 ECD (cat# 6604701 Beckman Coulter)	3µL CD20 ECD (cat# IM3607 Beckman Coulter)
3µL CD5 ECD (cat# A33096 Beckman Coulter)	
3µL CD7 ECD (cat# A70202 Beckman Coulter)	

16. Add the prepared antibodies to their respective tubes, vortex gently, and incubate at 4°C for 20 minutes in the dark

17. Wash the surface tubes with 2mL wash buffer (see Supplement 5) and centrifuge at 200g for 7 minutes at 4°C

18. Aspirate the supernatant, add 2mL of wash buffer, mix gently, and centrifuge at 240g for 5 minutes at 4°C

19. Aspirate the supernatant a second time and proceed to add 300µL ice cold 1x PBS

20. Acquire the samples on a flow cytometer (a Beckman Coulter Gallios in our case). Depending on time and reagent availability, single color compensation controls for the AML surface tubes should be run before acquiring the multi-color panels. This will most likely be required only once to set the compensation values for the panels

during analysis, provided the PMT voltages are saved with the initial protocol and utilized in subsequent experiments.

After acquiring the surface tubes, perform a brief analysis to determine where (if at all) any heterogeneity exists in the abnormal cells of the sample. Teasing out heterogeneity may be essential for determining a unique signaling pattern. If any heterogeneity is detected in the abnormal cells from the surface panels, employ these markers in the stimulation antibody panel. In addition to the antigens/markers used for targeting abnormal cells, including markers such as CD45, CD15, and CD64 will aid in the identification of any underlying normal cell population which, (if available) can serve as an effective positive or negative control for the abnormal population. These surface panels may also help weed out less useful markers to free up slots in the stimulation panel – markers that provide no useful separation may be dropped in favor of a less complex compensation profile.

After determining which antigens are expressed heterogeneously, construct antibody panels for use with the stimulation experiment. This will involve assembling two separate panels; one for the non-methanol treated ERK/S6/Akt samples, and a second for the 80% methanol treated STAT samples. The surface markers in the ERK panel can differ from those of the STAT panel due to the availability of suitable antibody/fluorophore combinations. The following is an example of panels we have run on past AML samples:

ERK Panel	**STAT Panel**
2µL pS6 PacificBlue (custom conjugate from Coulter)	5µL CD15 PacificOrange (cat# MHCD1530 Invitrogen)
5µL CD15 PacificOrange (cat# MHCD1530 Invitrogen)	2µL pStat1 AlexaFluor 488 (cat# 9174 Cell Signaling)
3µL pERK AlexaFluor 488 (cat# 4344 Cell Signaling)	5µL pStat5 PE (custom conjugate from Coulter)
4µL CD34 ECD (cat# IM2709U Beckman Coulter)	4µL CD34 ECD (cat# IM2709U Beckman Coulter)
2µL CD117 PE-Cy5.5 (cat# A66333 Beckman Coulter)	2µL CD117 PE-Cy5.5 (cat# A66333 Beckman Coulter)
2µL pAkt AlexaFluor 647 (cat# 4075 Cell Signaling)	3µL pStat3 AlexaFluor 647 (cat# 4324 Cell Signaling)
1µL CD45 AlexaFluor 700 (cat# 560566 BD)	1µL CD45 AlexaFluor 700 (cat# 560566 BD)

In this particular case, CD34 was the only examined antigen that had heterogeneous expression in the abnormal cells from these samples, leading to the omission of otherwise typical surface marker antibodies (e.g. CD13, CD14, CD16 and CD64).

Important: If antibodies such as CD13, CD16, or CD64 are to be included in the stimulation experiment, it is necessary to add them prior to the addition of formaldehyde to counter the

decrease in antibody sensitivity associated with fixing. In addition, some antibodies will not tolerate treatment with methanol, so optimization on a case-by-case basis will be necessary to determine antibody/methanol efficiency.

In order to determine how many tubes to prepare, it will be necessary to decide how many stimulants/growth factors will be in the experiment. This can vary based on the cell count, the volume of sample available, the phenotypic characteristics, and the frequency of the population of interest in the sample. Volume is a consideration only if less than 40µL of sample will be in each tube, as vortexing becomes less effective and as the effects of pipette error increase at lower volumes.

Once the antibody panels for the stimulation experiment have been composed and the number of tubes/stimulants has been decided on, proceed with the stimulation experiment.

21. Label sterile 12x75 5mL polypropylene tubes (cat# 352053 BD) with the names of the stimulants and timepoints selected for the experiment – see Supplement 6 for an example list of tubes/variables

22. Aliquot 3 million cells into each tube and place in a water bath at 37°C for 15 minutes

23. While the tubes are incubating, prepare the solutions found in Supplement 7 and 8 and plot out the stimulation timetable, using the example in Supplement 9 as a guide

24. Additionally, reconstitute the inhibitors and growth factors with 1x PBS while waiting for the samples to normalize at 37°C (see Supplements 10a and 10b for growth factor and inhibitor concentrations)

25. Based on the immunophenotype, determine if pre-fixation antibody exposure is required for antigens included in the stimulation antibody panel. Assemble the pre-fixation antibody master-mix if necessary. It is prudent to include more ‘extra’ tests than usual, n+2-5 tests depending on the number of tubes, as the experiment is time-based and to fall short of master-mix will disrupt the timing of the rest of the experiment.

26. Ensure all solutions required for the stimulation experiment are ready and at hand. This experiment can become fast-paced, especially if over 40 tubes are being processed by a single technician.

Timepoint	Stim	Fix	Triton
35 min	Add inhibitors	1:29:30	1:19:30
30 min	2:00:00	1:30:00	1:20:00
22 min	Add antibodies	-	-

15 min	1:45:30	1:30:30	1:20:30
7 min	1:38:00	1:31:00	1:21:00
3.5 min	1:35:00	1:31:30	1:21:30
2 min	1:34:00	1:32:00	1:22:00

27. Begin by starting the countdown timer at 2:05:00 and by adding the appropriate volume of each inhibitor to its respective tube while vortexing gently

28. At 2:00:00 on the timer, add each growth factor to its respective tube in the '30 min' group and vortex gently, returning the tubes to the 37°C water bath

29. At 1:52:00 add the correct volume-per-test of the pre-fix antibody master-mix to **all** tubes (if necessary) and vortex to mix. This panel may differ between the methanol and non-methanol treated tubes, so ensure the correct panel is added to the correct tubes.

30. At 1:45:30 add the growth factors to the '15 min' group and vortex gently

31. At 1:38:00 add the growth factors to the '7 min' group and vortex gently

32. At 1:35:00 add the growth factors to the '3.5 min' group and vortex gently

33. At 1:34:00 add the growth factors to the '2 min' group and vortex gently

34. At 1:32:00, begin fixing by adding 65µL of the 10% formaldehyde solution (Supplement 7) to each of the '2 min' tubes while vortexing

35. After vortexing, place the tubes in a rack outside the waterbath at 25°C

36. Continue adding fixative to the remaining tubes according to the stimulation timetable in Supplement 9

37. After fixative has been added to all the tubes, place the rack in the dark for 10 minutes, starting at the time that the first '2 min' tube was fixed, ensuring that the tubes remain at 25°C

38. After 10 minutes has elapsed, add 1mL 0.01% Triton X-100 solution (Supplement 8) to each tube, following the time it took to fix each set of tubes refer (Supplement 9)

39. Put the tubes back into the 37°C waterbath for 15 minutes in the dark

40. After 15 minutes, remove the tubes and add 2mL ice cold wash buffer (Supplement 5) to each

41. Centrifuge the tubes at 800g for 6 minutes at 4°C

42. Remove the tubes from the centrifuge and place on ice while aspirating the supernatant

43. At this point, the tubes should be separated into either the 'methanol treated' (i.e. STAT) group or the 'non-methanol treated' (i.e. ERK/S6/AKT) group; each group is processed differently. *For the remainder of this protocol a distinction will be made regarding how each set of tubes should be processed.*

44. Add 50µL of staining buffer (Supplement 4) to each of the 'non-methanol treated' tubes and keep on ice for 15 minutes

45. Meanwhile, resuspend the 'methanol' tubes in 2mL of ice cold 1x PBS

46. Centrifuge the 'methanol' tubes at 800g for 6 minutes and aspirate the supernatant

47. Place the tubes on ice and resuspend the pellet by adding 200µL ice cold 1x PBS to each 'methanol' tube

48. To each of these 'methanol' tubes, add 800µL of ice cold methanol (cat# MX0475-1 EMD) dropwise while vortexing vigorously

49. Incubate the 'methanol' tubes at 4°C for approx. 1 hour

50. While the 'methanol' tubes are incubating, assemble the antibody panels for the stimulation experiment based on the panels created earlier from the results of the immunophenotyping experiment

51. Add the correct volume of 'ERK panel' antibodies to the 'non-methanol' tubes and incubate at 4°C for approx. 45 minutes

52. After the appropriate incubation, remove both the 'methanol' and 'non-methanol' treated samples from 4°C and add 2mL ice cold 1x PBS to each tube

53. Centrifuge all tubes at 800g for 6 minutes at 4°C and aspirate the supernatant

54. Repeat the wash and spin steps once more while keeping the samples at 4°C

55. Aspirate the supernatant from all tubes and add 50µL staining buffer to the 'methanol' treated tubes incubate on ice for 15 minutes in the dark

56. Add 250µL ice cold 1x PBS to the 'non-methanol' treated samples while keeping them on ice

57. Vortex the 'non-methanol' tubes to ensure they are resuspended and keep in the dark until acquisition on a flow cytometer

58. Add the correct volume of ‘STAT panel’ antibodies to the ‘methanol’ tubes and incubate at 4°C for approx. 45 minutes

59. Add 2mL ice cold wash buffer to each of the ‘methanol’ tubes and centrifuge at 800g for 6 minutes at 4°C

60. Aspirate the supernatant and repeat the wash and spin steps while keeping the samples at 4°C

61. Aspirate the supernatant and resuspend in 250µL ice cold 1x PBS

62. Vortex the ‘methanol’ tubes to ensure the samples are resuspended and acquire on a flow cytometer

After acquiring the data, the user should begin to analyze the results. This involves using post-acquisition analysis software such as FCS Express or FlowJo. The goal of analysis is to extract meaningful statistics from the mass of data generated by this experiment. These statistics concern the response of the phospho-proteins to growth factors or inhibitors, and while other statistics like percent CD34 positive may be useful, they aren’t the goal of this analysis. This lab uses three main statistics in regard to phospho-proteins in several discrete immunophenotypically defined cell populations:

Median - the median fluorescence intensity (MFI) of the phospho-protein in a specified population

Percent Responding - the percent of events that are above a fluorescence threshold set from the inhibitor tube corresponding to the phospho-protein in question

Median of the Percent Responding - the median fluorescence intensity of the cells above the threshold set in the previous statistic

All of these are raw statistics that the analysis program produces, but in order to be effective they have to be compared to internal controls. This is achieved by looking at the ratio of signal to noise in a certain channel, using lymphocytes as the internal control. The lymphocyte population serves as an excellent internal control because it shows no response to any growth factors used to stimulate the samples. In order to obtain the signal to noise ratio for phospho-STAT5 in the monocytes, for example, it is necessary to divide the MFI of the responding monocytes in the STAT5 channel by the MFI of the lymphocytes in the STAT5 channel. This produces the desired normalized statistic. This technique can be applied to any population in any channel desired, as long as there is an internal negative control.

Supplemental Recipes/Protocols

1a. Ammonium Chloride Lysis Solution (10x)

80.2g NH_4Cl (1.5M) (cat# A-0171 Sigma)

8.4g $NaHCO_3$ (100mM) (cat# S-4772 Sigma)

3.7g disodium EDTA (10mM) (cat# E-5134 Sigma) **or** 3.66g tetrasodium EDTA (8.2mM) (cat# ED4SS Sigma)

Add H_2O to 900mL

Adjust pH to 7.4 with 1N HCl or NaOH

Add H_2O to 1L

Store at 4°C

1b. PBS (10x)

1600g NaCl (cat# S271-50 Fisher)

230g $Na_2HPO_4*7H_2O$ (cat# S2429-3KG Sigma)

40g KCl (cat# P5405 Sigma)

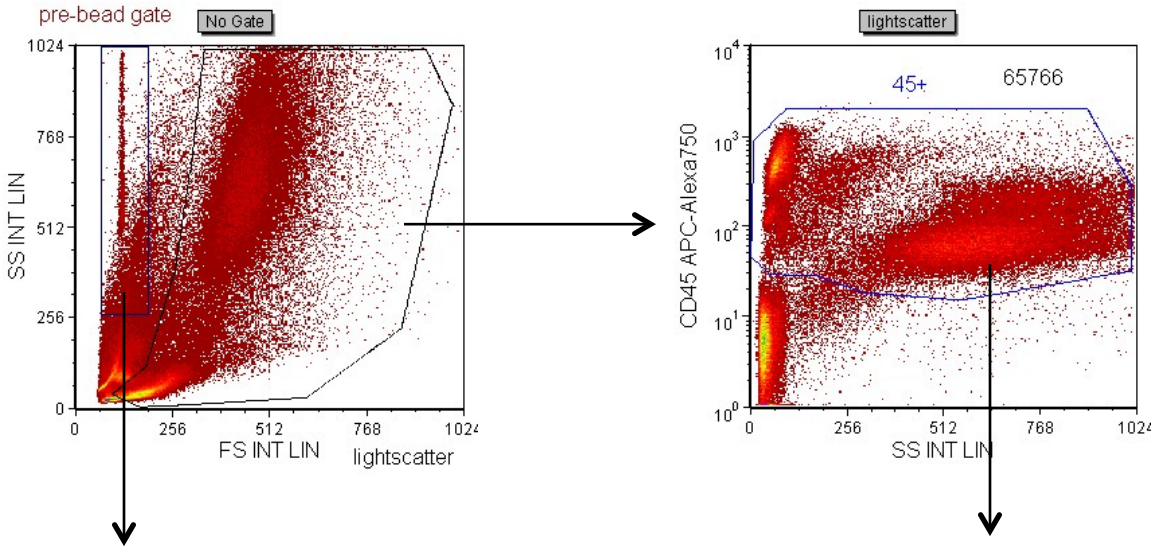
40g KH_2PO_4 (cat# P5655 Sigma)

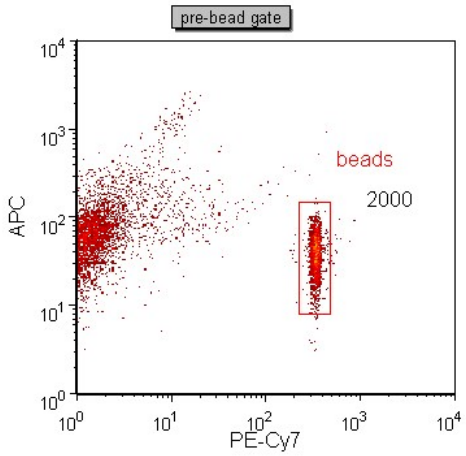
Add 20L DI water and stir until the components are completely dissolved

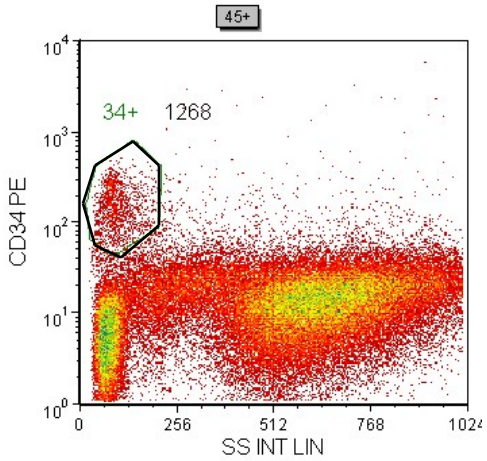
Store at room temperature

2. Determination of cell number per unit volume using Spherotech beads

When the proper antibodies and beads are added to the sample and the sample is lysed, run the tube on a flow cytometer equipped with both a red (640nm) and blue (488nm) laser. Set up a gating scheme that is similar to the following:







Once the gates are drawn, set statistics so the number of events in each gate is visible and set up a stop condition on the "bead" gate of 2000 events. Proceed to collect the sample until it reaches the 2000 bead limit.

The beads from Spherotech are at a known concentration of $1x10^6$ per mL, so by adding equal volumes of beads and sample, enumeration of the cells in the sample is possible. This is achieved by dividing the number of live cells (CD45+ = 65766) by the number of beads collected (2000). The resulting number is 32.883, which is how many 'millions' of cells are in one milliliter of sample. The final concentration of 45+ cells in this sample is $3.29x10^6$ cells/100μL. A determination of the 34+ cell percentage is also possible by dividing the 34+ cell count by the 45+ cell count and multiplying by 100 ((1268/65766)*100 = 1.928% 34+). The 34+ concentration can be obtained by multiplying $3.2x10^6$ cells/100μL by 0.01928 ($6.3x10^4$ cells/100uL).

3. Determining the correct initial cell number in stimulation sample tubes

Ensuring the appropriate number of cells remain in the tube following the various washes and lysing a protocol entails is essential for statistical significance and acquisition time considerations. The following is a brief guide to help compensate for inevitable cell loss.

A good number to shoot for, at least at first, is 50,000 'cells of interest' before starting the protocol. This ensures that, even given a 10-fold reduction in cells, there will be 5,000 cells analyzed by the cytometer at the end of the experiment. If the population of interest is rare (below 1%) starting with this many cells may be difficult and an increase of starting material (blood), a decrease in expected yield, or adjustments to aspiration technique may be necessary. If, on the other hand, the population is abundant (abnormal cells in an AML sample) the initial volume of blood may be reduced to facilitate more tubes in the experiment. A consideration: 'cells of interest' also include any internal controls that are necessary for the experiment. In the above example of AML, the lymphocytes would be considered a 'population of interest' as a negative control, thereby warranting attention to the final number of cells.

The majority of 'lost cells' are disposed of accidentally when aspirating the supernatant after centrifuging the sample. Protocols with more wash steps, namely the addition of methanol, will result in increased cell loss; compensating by adding more blood at the beginning is a way around this. It may be necessary to analyze only 2,000 (or fewer) cells at the end of an experiment to facilitate the inclusion of desired variables, given a limited initial sample volume (as is often the case with clinical samples).

Important: If blood is added to or subtracted from the experiment, be sure to adjust the formaldehyde and inhibitor volumes respective to the volume of blood in the tubes.

4. Staining Buffer

The staining buffer we use is comprised of 25% Rabbit serum (cat# ab7487 Abcam), 25% Mouse serum (cat# ab7486 Abcam), and 50% sterile PBS. The two sera are mixed in an equal ratio and then 625µL aliquots are placed in cryovials (cat# 430488 Corning) and frozen at -80°C. When we are ready to stain a sample we remove an aliquot and add 625µL of sterile PBS, mix well, and add 50μL of the buffer to our samples.

5. Wash Buffer

The wash buffer we use is comprised of 2%BSA (cat# A7030-500G Sigma-Aldrich) (weight/volume) in 1x PBS. After the BSA is added to the PBS, the solution is passed through a 0.2µm filter (cat# 567-0020 Nalgene) and kept at 4°C.

6. Example Stimulation Experiment Tube List

Control Tubes

Unstimulated	MeOH Unstimulated
U0126 treated	MeOH U0126 treated
Rapamycin treated	MeOH Rapamycin treated
GDC0941 treated	MeOH GDC0941 treated
Sorafenib treated	MeOH Sorafenib treated

ERK Tubes

SCF 2min	Flt3L 2min	GMCSF 2min	GCSF 2min
SCF 3.5min	Flt3L 3.5min	GMCSF 3.5min	GCSF 3.5min
SCF 7min	Flt3L 7min	GMCSF 7min	GCSF 7min

SCF 15min	Flt3L 15min	GMCSF 15min	GCSF 15min
SCF 30min	Flt3L 30min	GMCSF 30min	GCSF 30min

MeOH treated STAT Tubes

GMCSF 3.5min	GCSF 3.5min	MCSF 3.5min	IFN-g 3.5min
GMCSF 7min	GCSF 7min	MCSF 7min	IFN-g 7min
GMCSF 15min	GCSF 15min	MCSF 15min	IFN-g 15min
GMCSF 30min	GCSF 30min	MCSF 30min	IFN-g 30min

7. Formaldehyde

The fixing agent is made from 16% formaldehyde in ampules (cat# 18814 Polysciences) and sterile 1x PBS. To make 1mL of 10% formaldehyde, add 375µL 1x PBS to 625µL 16% formaldehyde. This is usually enough to fix 15 100µL samples.

8. Triton X-100

The permeablization agent is made by combining 150µL 10% Trition X-100 solution (cat# 28314 Thermo Scientific) with 14.85mL sterile 1x PBS. 15mL is enough for 15 samples.

9. Stimulation Timetable

In order to minimize the effects of human error due to timing, we have implemented a strategy for staggering the stimulation timepoint to enable accurate lengths of growth factor exposure. The goal is to offset the fixation time by a comfortable margin (allow 10 seconds per tube at a given timepoint – with practice this may be reduced to around 6 seconds per tube) and then extrapolate the correct time at which to stimulate. See the example below with 5 timepoints and 3 tubes stimulated per timepoint:

Timepoint	Stim	Fix	Triton
35 min	Add inhibitors	1:29:30	1:19:30
30 min	2:00:00	1:30:00	1:20:00

22 min	Add antibodies	-	-
15 min	1:45:30	1:30:30	1:20:30
7 min	1:38:00	1:31:00	1:21:00
3.5 min	1:35:00	1:3 1:30	1:21:30
2 min	1:34:00	1:32:00	1:22:00

The times on this table correspond to a timer set to count down from 2 hours. If any antibodies need to be added before fixing, we add them at the 22 minute mark (at 1:52:00) from a master-mix prepared beforehand.

10a. Growth Factors concentrations

The following is a table of growth factor sources and concentrations that are used in the stimulation experiment. The final concentration is the concentration of growth factor in the 100µL of blood during the stimulation.

Name	Freezer Concentration	Working Concentration	Final Concentration	Vendor	Cat #
SCF	10µg/mL	100ng/100µL	10ng/100µL	R+D	255-SC-010
Flt-3L	25µg/mL	500ng/100µL	50ng/100µL	R+D	308-FKN-025
GM-CSF	10µg/mL	100ng/100µL	10ng/100µL	R+D	215-GM-010
G-CSF	100µg/mL	100ng/100µL	10ng/100µL	ORF	01-AA070
M-CSF	100µg/mL	100ng/100µL	10ng/100µL	ORF	01-A0220
IFN-gamma	100µg/mL	500ng/100µL	50ng/100µL	ORF	01-A0060
Il-3	10µg/mL	100ng/100µL	10ng/100µL	R+D	203-IL-010

10b. Inhibitor concentrations

The following is a table of inhibitor sources and concentrations that are used in the stimulation experiment.

Name	Freezer Concentration	Final Concentration	Vendor	Cat #
U0126	10mM	100µM	EMD	662005-1MG
Rapamycin	10mM	1µM	EMD	553210-1MG
GDC0941	5mM	50µM	Axon Medchem	Axon 1377
Sorafenib	100mM	1mM	TRC	S676850