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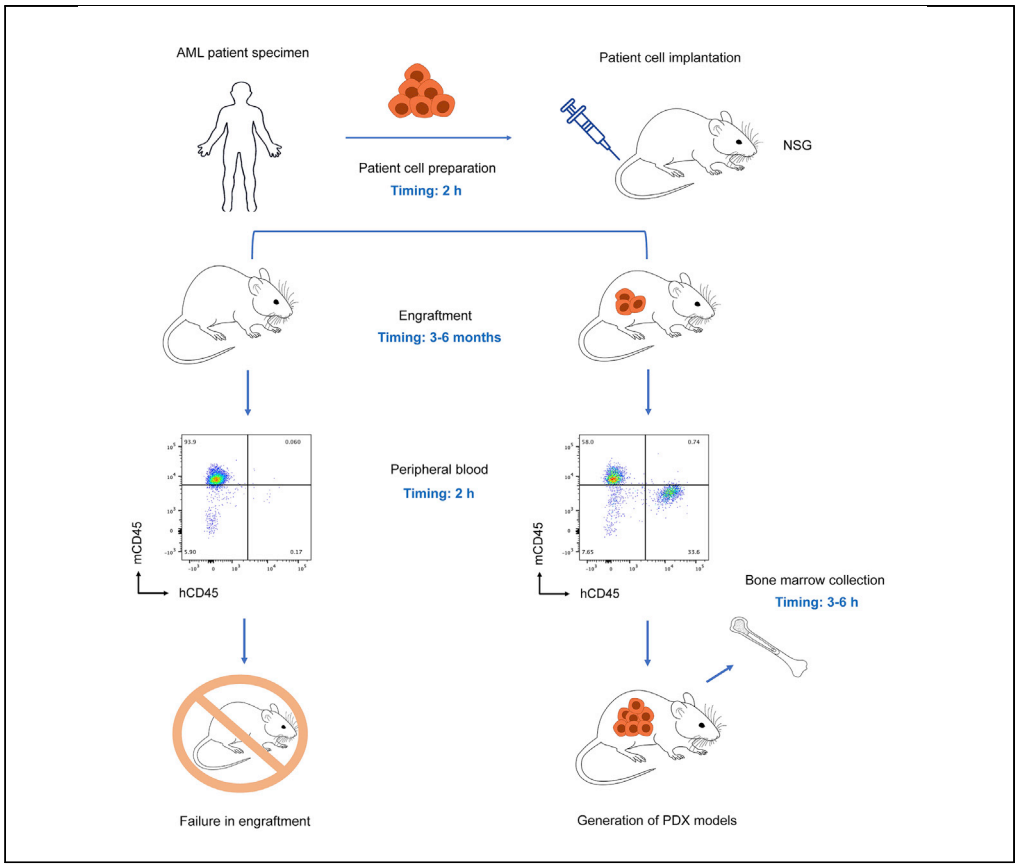
Protocol

An Improved Protocol for Establishment of AML Patient-Derived Xenograft Models



Patient-derived xenografts (PDXs) are the most valuable tool for preclinical drug testing because they retain the genetic diversity and phenotypic heterogeneity of the original tumor. Acute myeloid leukemia (AML) remains difficult to engraft in immunodeficient mice. This is particularly true for long-term frozen patient specimens. This protocol is designed to establish PDXs of human AML with improved engraftment rates. The optimized approach increases the viability of patient cells before implantation, efficiently monitors *in vivo* engraftment and maximizes bone marrow collection.

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HIGHLIGHTS

This protocol describes the establishment of AML patient-derived xenograft mouse models

Increased viability of injected cells is critical for high engraftment rate in mice

Effective collection of bone marrow is a key step in the process of PDX establishment

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An Improved Protocol for Establishment of AML Patient-Derived Xenograft Models

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SUMMARY

Patient-derived xenografts (PDXs) are the most valuable tool for preclinical drug testing because they retain the genetic diversity and phenotypic heterogeneity of the original tumor. Acute myeloid leukemia (AML) remains difficult to engraft in immunodeficient mice. This is particularly true for long-term frozen patient specimens. This protocol is designed to establish PDXs of human AML with improved engraftment rates. The optimized approach increases the viability of patient cells before implantation, efficiently monitors *in vivo* engraftment, and maximizes bone marrow collection.

For complete details on the use and execution of this protocol, please refer to Salik et al. (2020) and Lynch et al. (2019).

BEFORE YOU BEGIN

All mouse experiments must be performed with the approval of an Animal Care Committee that is relevant to your research institution. Primary AML patient specimens carrying MLL-AF9, MLL-AF10, MLL-AF1q, or 9p deletion used in this protocol were obtained from the Sydney Children's Tumor Bank Network and were consented for research purpose. Ethical approval was obtained from the Sydney Children's Hospitals Network Human Research Ethics Committee.

All reagents must be made up and sterilized through a 0.45 μm filter prior to use.

Preparation of Reagents and AML Patient Specimens

Timing: 10 min

1. Prepare 20% FBS-containing RPMI-1640 medium and 0.25% FBS-containing PBS.
2. Before starting, keep AML patient samples on dry ice.
3. Set the centrifuge temperature to 4°C.

⚠ CRITICAL: AML patient samples must remain frozen on dry ice prior to thawing.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC anti-human CD45	BD Bioscience	555485
FITC anti-mouse CD45	BD Bioscience	553080

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Buffers, and Reagents		
Fetal bovine serum (FBS)	Thermo Fisher Scientific	10099133
Trypan blue solution	Thermo Fisher Scientific	15250061
RPMI-1640 medium	Thermo Fisher Scientific	22400089
Penicillin-streptomycin-glutamine (100×)	Thermo Fisher Scientific	10378016
BD Pharm Lyse RBC lysing buffer	BD Bioscience	5558999
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
Dulbecco's phosphate-buffered saline	Sigma-Aldrich	D5652
Biological Samples		
Human: primary AML patient specimens carrying MLL-AF9, MLL-AF10, MLL-AF1q, or 9p deletion	This study	N/A
Experimental Models: Organisms/Strains		
Mouse: NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG)	Australian BioResources Mossvale	N/A
Software and Algorithms		
FlowJo Software	TreeStar	In house license
BD FACSDiva Software	BD Bioscience	In house license
GraphPad Prism 8	GraphPad Software	In house license
Other		
0.5 mL Terumo insulin syringes with 27G × 0.51 inch needle	Medshop Australia	SKU TER00141
24-well tissue culture plate with flat bottom	Merck	CLS3524
50 mL centrifuge tube	Life Sciences	430829
15 mL centrifuge tube	Life Sciences	430791
1 mL Cryo.s with screw cap	Greiner Bio-One	123263
1.5 mL Eppendorf Safe-Lock microcentrifuge tube	Merck	T9661
5 mL round-bottom polystyrene tube with cell-strainer cap	Life Sciences	352235
0.5 mL MiniCollect tube K3E K3EDTA	Greiner Bio-One	450530
Rediwipe	Cello Paper	ARW 3233
10 mL Corning Costar Stripette serological pipettes	Merck	CLS4488
25 mL Corning Costar Stripette serological pipettes	Merck	CLS4489
EASYstrainer Cell Strainer 40 µm	Greiner Bio-One	542040
Millex-HP Syringe Filter unit 0.45 µm	Millipore	SLHP033RS
Hemocytometer (counting chamber)	ProSciTech	SVZ4NIOU
Nalgene Cryo 1°C Freezing Container	Thermo Scientific	5100-0001
CKX41 Inverted Microscope	Olympus	CKX41SF
Coors porcelain mortar	Merck	Z247464
Coors porcelain pestle	Merck	Z247502
Stainless steel forceps	Merck	Z168696
Dissecting scissors	Sigma-Aldrich	Z265969

STEP-BY-STEP METHOD DETAILS

Preparing AML Patient Cells

Timing: 2 h

Patient samples frozen in DMEM supplemented with 20% FBS and 10% DMSO have been stored in liquid nitrogen for 10–30 years. Thus, the thawing process is one of the most critical steps because it determines the viability of AML patient cells and the ultimate success of cell engraftment in mice.

1. Thaw frozen patient cells rapidly in a water bath at 37°C (<1 min).
2. Immediately transfer the thawed cells dropwise into a large volume of pre-warmed 20% FBS-containing RPMI-1640 medium in 50 mL centrifuge tube.
3. Filter the cell suspension through a 40 μm Cell Strainer to remove cell clumps and debris.
4. Centrifuge the tubes at 250 × *g* for 5 min at 4°C.
5. Discard the supernatant and resuspend the cell pellet in 500 μL of 0.25% FBS-containing PBS.
6. Count the number of cells in a hemocytometer.

Note: Perform a 1:10 dilution cell count by resuspending in 5 μL of cell suspension with 45 μL trypan blue

7. Determine the number of viable patient cells to be injected into NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice.

Note: Divide the total number of viable cells by the number of mice to be injected to calculate how many cells will be injected into each mouse.

- The mouse number is largely dependent on the total number of viable cells.
- If possible, inject more than 1 × 10^6 viable cells per mouse, for at least three individual NSG mice. Otherwise, a minimum of 5 × 10^5 cells will need to be injected per mouse.

8. Transfer the cell suspension to an Eppendorf tube.
9. Keep the Eppendorf tubes on ice for injections.
10. Perform intravenous (IV) injections in NSG mice.

Note: Prior to injection, warm mice under a heat lamp till tail vein dilation (5–10 min).

- Place mouse cages in the front of a heat lamp and no closer than 15 cm from the lamp.
- Monitor all mice being heated frequently. If mice are observed to be inactive, panting, sweaty, or have red extremities, remove cages from the heat before mice may experience heat exhaustion.

Note: Load a syringe with patient cells and inject cells into the lateral tail vein using a 27- or 28-gage sterile needle.

- Ensure there are no air bubbles in the syringe.
- The maximum recommended injection volume is up to 5 mL/kg.
- IV injections should be performed by experienced researchers.

⚠ CRITICAL: Make sure that the cells are kept on ice and are injected immediately after preparation. Troubleshooting

Monitoring Engraftment of Patient Cells in Mouse Peripheral Blood

Timing: 15 min

After mice are injected with patient cells, assess engraftment of human leukemic cells in mouse peripheral blood starting from 3–4 weeks of implantation. The peripheral blood will be collected and analyzed once per week over a period of 6 months.

Note: AML patient cells often take 3–6 months to engraft depending on the number and quality of viable cells injected in NSG mice.

11. Prepare 1× RBC (red blood cell) lysis buffer.
    a. Perform a 1:10 dilution of BD Pharm RBC Lysing solution (10×) with ddH_2O.
    b. 700 μL of 1× RBC lysis buffer is used for one sample (~20–40 μL of peripheral blood).
    c. Store the RBC lysis buffer at room temperature (~20°C).
12. Prepare a cocktail of antibodies for FACS analysis.
    d. Add 4 μL of human CD45 (hCD45)-APC and 2 μL of mouse CD45 (mCD45)-FITC to 94 μL PBS (per reaction/sample).
        - mCD45 has a final concentration of 2 ng/mL.
        - hCD45 has a final concentration of 1.25 ng/mL.
    e. Keep on ice until further use.

Processing Peripheral Blood to Monitor Engraftment

Timing: 2 h

About one month after injection, patient cell engraftment in mouse peripheral blood will be monitored through measuring the profile of $hCD45^+$ and $mCD45^+$ cells using FACS analysis.

13. Collect about 3 drops (~ 20–40 μL) of tail vein blood into a 0.5 mL MiniCollect tube (K3E K3EDTA).

Note: The MiniCollect tube prevents coagulation of the blood as it contains EDTA.

Note: Tail vein blood collections are performed by experienced researchers.

14. Add 1 mL of PBS in the MiniCollect tube and then transfer it to an Eppendorf tube.
15. Centrifuge the Eppendorf tube for 8 min at 250 × *g* at 4°C. Discard the supernatant.
16. Add 700 μL of 1× RBC lysis buffer, gently mixing each tube immediately. Troubleshooting
17. Incubate in the dark at ~20°C for 15 min.

Note: Do not incubate for longer than 15 min as it could lyse white blood cells.

18. After incubation, top up with PBS to ~1.4 mL and invert tube to mix.
19. Centrifuge at 250 × *g* for 8 min at 4°C. Discard the supernatant.
20. Resuspend the cell pellet in 95 μL PBS and add 100 μL of antibody cocktail to each tube and mix well.
    a. Vortex briefly to mix well.
21. Incubate the tube in the dark for 35 min at 4°C. Troubleshooting
22. Top up the cell and antibody mixture with PBS (~1 mL) and invert tube to mix.
23. Centrifuge at 250 × *g* for 8 min at 4°C. Discard the supernatant.
24. Resuspend one sample aliquot in 200 μL PBS and filter through 35 μm blue filter cap into a polypropylene FACS tube to proceed with FACS analysis using a multicolor flow cytometer (e.g., BD FACSCanto II).
25. Analyze flow cytometry data using BD FACSDiva software.
    a. Unstained samples and single stained samples are used to set appropriate photomultiplier tubes voltages that have clear positive and negative populations.

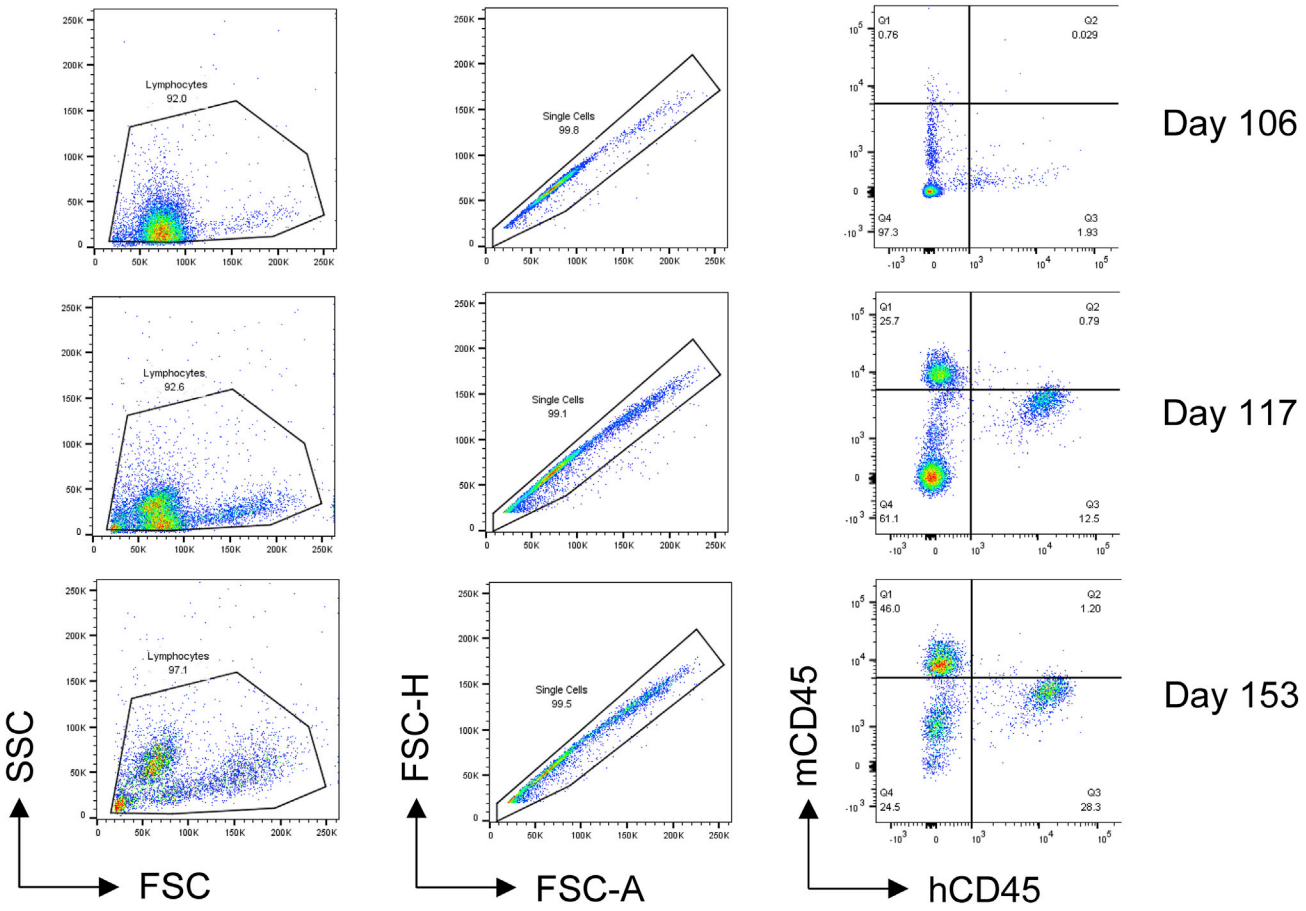

Figure 1. Flow Cytometry Plots Depicting Percentages of hCD45 versus mCD45 in the Peripheral Blood of NSG Recipient Mice at the Indicated Days After Implantation

26. Analyze the results using the FlowJo software (Figure 1).

Harvesting Bone Marrow from AML Patient-Derived Xenograft Models

Timing: 3–6 h

Effective collection of bone marrow is an integral part in the process of PDX mouse model establishment and use. This step is to ensure the maximum bone marrow collection from a PDX mouse by harvesting spinal cord and femurs/tibias.

⚠ CRITICAL: To preserve tissue cell viability, it is important to collect and process the tissue samples within 6 h after surgical resection.

Use scissors and forceps with curved ends.

27. Upon confirmation of patient cell engraftment in peripheral blood, euthanize the mouse with carbon dioxide asphyxiation or an alternative method approved in accordance with institutional animal care and use guidelines.
28. Place the mouse onto a sterile surgical pad in a sterile hood.

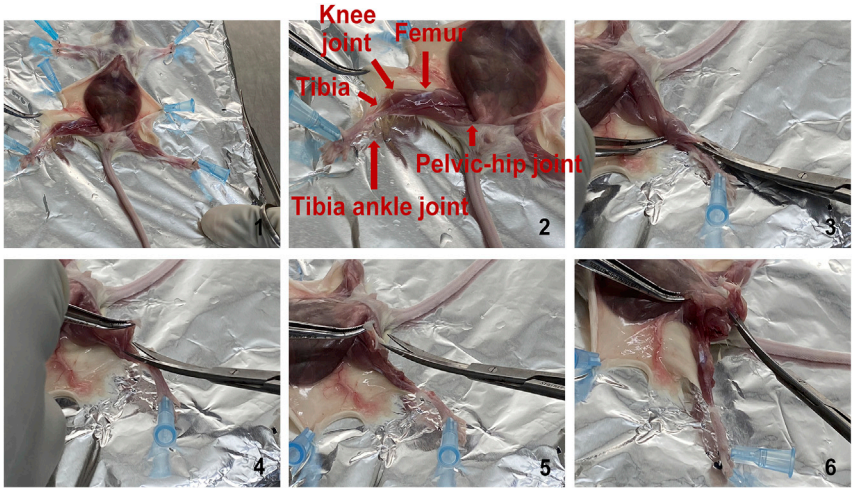

Figure 2. Dissection of the Mouse Femur and Tibia

29. Pin the mouse in a supine position to a dissection board by putting a pin through each of the four paws. Spray the entire body with 80% ethanol.
30. Open the lower abdominal cavity with sterile scissors and remove the surface muscles to find the pelvic-hip joint and tibia ankle joint (Figure 2).
31. Cut off the hind leg above the pelvic-hip joint and below the tibia ankle joint with sharp sterile scissors.
32. Remove the residual muscle tissues surrounding the femur and tibia with sterile forceps and scissors.

Note: Increased spleen size is an important sign of leukemia. Mice with significant engraftment of leukemic cells often have an enlarged spleen.

33. Femurs and tibias: Cut along the inner side of bone up to the pelvic-hip joint until the incision cannot be continued. Attempt to cut as close to the bone as possible. Then cut along the top of the bone structures. Cut at the hip joint, and then cut the distal tibia ankle joint. Then remove the bones and place them on a rediwipe and proceed to remove as much residual muscle as possible. Cut the bony structure at the knee joint and place in PBS. Repeat for the other femur/tibia.
34. Spine: Turn mouse over into the prone position, re-pin, and spray 80% ethanol on the back. Make an incision in the skin over the base of the spine and cut right up till the head and down to the tail. Cut along the left side of the spinal cord until the cut cannot continue any further, then repeat along the right side. Cut underneath the spinal cord removing as much tissue as possible. Cut at the top of the spinal cord and then the bottom at the tail. Place the spinal cord on a rediwipe and proceed to remove as much residual muscle and connective tissue as possible, cutting in one motion alongside bone, and then place in PBS.
35. In a biological safety cabinet, cut and wipe away all muscle tissue from the spinal cord and femurs on a rediwipe, and then place tissue samples in a petri dish submerged in a small amount of PBS.
36. Pour a small amount of fresh PBS into a mortar and pestle.
37. Cut the spinal cord into small pieces and place them into the mortar.

Note: Place scissors and forceps on a petri dish lid to avoid contamination.

38. Crush the spinal cord, and then place femurs and tibias into the mortar and continue crushing until the fibrous texture is achieved.

39. Using a P1000, pipette in 1 mL PBS from the mortar into a 50 mL centrifuge tube with a 40 μm cell strainer to remove debris. Pipette PBS from the surface, trying not to pipette any bony fibers.
40. Pour some fresh PBS into the mortar and crush until the remains are white and fibrous.
41. Pipette PBS from mortar into the tube using stripettes.

Note: Using stripette will speed up the process.

Note: If the filter becomes blocked, replace with a new filter and pipette any remaining liquid from the old filter into the new filter.

42. Pour some fresh PBS into the mortar and pipette the mixture into the 50 mL centrifuge tube with a stripette.
43. Repeat steps 44 and 45 until the liquid in tube reaches ~40 mL.
44. Place the centrifuge tubes on ice.
    c. Mice from same cage: wipe away all remaining fibrous tissue with rediwipe and rinse mortar and pestle with PBS.
    d. Mice from different cages: Wash and wipe mortar, pestle, scissors and forceps with 80% ethanol and rinse with PBS.
45. Repeat steps 39–47 for all samples, keeping all tubes on ice.
46. Spin all the tubes together at 250 × *g* for 5 min at 4°C.
47. Remove the supernatant carefully and resuspend the pellet in 1 mL of 1× RBC lysis buffer.
48. Incubate for 7 min at ~20°C, then inactivate lysis buffer by topping up the tube to 50 mL with PBS.
49. Centrifuge at 250 × *g* for 5 min at 4°C.
50. Remove the supernatant and resuspend the pellet in 1 mL freezing media.

Note: Sterile freezing media composes of 10% DMSO, 50% FBS and 40% RPMI-1640 medium.

51. Pipette 5 μL of the cell media into an Eppendorf tube for counting.
    a. Dilute 5 μL cell media with trypan blue.
        - Create a 1:100 dilution by adding 495 μL trypan blue to 5 μL of cell suspension.
52. Count cells using hemocytometer by putting 10 μL of solution under the coverslip.

Note: Make sure to wipe hemocytometer with ethanol and wipe dry with chemwipe.

53. After the cell count, determine the quantity of cells to freeze down for later use. Troubleshooting

Note: Do not put more than 10^7 cells per cryovial to prevent freeze-thaw cycles and reduce cell viability.

Note: Aliquot a small number of cells (e.g., 2–5 × 10^5) in several cryovials for FACS or other downstream analysis.

54. Transfer the remaining cells into cryovials (1 mL/vial) to freeze at −80°C freezer for 18–24 h in Nalgene Cryo 1°C Freezing Container, and then transfer to liquid nitrogen on the following day.

⚠ CRITICAL: Be aware of the potential presence of human pathogens. Be sure to adhere to appropriate biosafety protocols when handling human tissues. In addition, appropriate antibiotics should be added to the media to prevent bacterial contamination during tissue processing.

Table 1. Summary of a Pilot Study Engrafting AML Patient Specimens in NSG Mice

AML Patient Specimen	Engraftment	Patient Cells Injected per Mouse	Days Post-injection for BM Collection	BM Cells Harvested
MLL-AF1q	Yes	5.7×10^6	165	4.72×10^7
	Yes	5.7×10^6	165	4.40×10^7
	Yes	5.7×10^6	165	5.25×10^7
9p deletion	Yes	7.0×10^6	156	4.60×10^7
	Yes	7.0×10^6	156	5.10×10^7
	Yes	7.0×10^6	156	5.35×10^7
MLL-AF9	Yes	1.6×10^6	87	2.16×10^7
MLL-AF10 (Relapse)	No	1.7×10^6	147	n/a
MLL-AF10 (Diagnosis)	No	7.1×10^5	147	n/a

EXPECTED OUTCOMES

This protocol describes the establishment of AML patient-derived xenograft (PDX) mouse models for *in vivo* preclinical drug testing. Using this protocol, our pilot study has achieved a 60% engraftment rate in NSG mice (Table 1). There are three major steps optimized to improve *in vivo* engraftment of primary AML patient cells. This includes methods for increasing the viability of patient cells in the thawing process, monitoring *in vivo* engraftment efficiently and maximizing bone marrow collection from PDX mice. AML patient cells often take 3–6 months to engraft depending on the number and quality of viable cells injected in NSG mice. An optimal thawing process determines the quantity and quality of patient cells and increases the success rate of PDX model establishment.

LIMITATIONS

The major limitation of this protocol is that about 40% of primary AML patient samples fail to engraft in NSG mice. This is not surprising as AML has frequently been reported to be a difficult-to-engraft model. In particular, the use of frozen patient cells reduces cell viability and limits availability of viable cells to be transplanted into mice. Fresh patient samples or increased viable cells will substantially improve the success rate of a PDX model in this protocol.

TROUBLESHOOTING

Problem

There is low viability of patient cells after thawing.

Potential Solution

This may happen due to the thawing condition of patient cells as they are very sensitive. To avoid this situation, we recommend using 20% FBS and to keep cells on ice during the whole procedure.

Problem

There are no $hCD45^+$ peripheral blood cells observed in FACS analysis.

Potential Solution

There are several potential reasons: 1) not enough primary antibodies are used in flow cytometry; 2) the incubation period with antibodies is not long enough; 3) gating is incorrect and voltages are inappropriate; and/or 4) there is a failure of patient cell engraftment.

Problem

There is red blood cell residue in the FACS samples.

Potential Solution

A major reason for this problem may be that RBC lysis buffer is not working at its optimal condition. Ammonium chloride (NH4Cl) is the active agent in RBC lysis buffer and sufficient NH4Cl is required to remove the RBCs. However, the number of red blood cells within blood samples may vary. Thus, the volume of RBC lysis buffer will need to be further optimized, depending on the volume of blood to be collected.

Problem

There is low yield of harvested bone marrow cells.

Potential Solution

This may be due to several factors such as 1) not keeping samples on ice, 2) leaving the samples out for too long thus causing the low viability of PDX cells, and 3) not removing all muscles around the bone and joint prior to grinding, leading to cells being trapped between the muscles. It is therefore important to complete the harvesting procedure as quickly as possible and to sufficiently remove the surrounding tissues in order to increase the yield of harvested bone marrow cells.

Problem

There is a failure of patient cell engraftment in NSG mice.

Potential Solution

The number of viable patient cells injected is critical for achieving engraftment in NSG mice. The number of primary patient cells below the threshold, e.g., 5×10^5 cells per mouse, could be taken into account. If there are no mice engrafted with AML patient cells, the number of cells injected will need to be increased. This may also be caused by technical reasons during injection such as speed and force of injection. Furthermore, limited number of patient cells may be due to cell leakage during injection or the needle is too superficial to the site of injection leaving a subcutaneous bubble at the site of injection, thus losing cells.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jenny Y. Wang, Ph.D., jenny.wang@unsw.edu.au.

Materials Availability

No new mouse lines were generated in this study. NSG mice were obtained from Australian BioResources.

Data and Code Availability

No new data or code were generated for this study.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, J.Y.W.; Investigation, N.H. and J.Y.; Writing – Original Draft, N.H.; Writing – Review & Editing, J.Y.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Salik, B., Yi, H., Hassan, N., Santiappillai, N., Vick, B., Connerty, P., Duly, A., Trahair, T., Woo, A.J., Beck, D., Liu, T., Spiekermann, K., Jeremias, I., Wang, J., Kavallaris, M., Haber, M., Norris, M.D., Liebermann, D.A., D'Andrea, R.J., Murriel, C., and Wang, J.Y. (2020). Targeting RSPO-LGR4 signaling for leukemia stem cell eradication in acute myeloid leukemia. Cancer Cell *38*, 263–278.

Lynch, J.R., Salik, B., Connerty, P., Vick, B., Leung, H., Pijning, A., Jeremias, I., Spiekermann, K., Trahair, T., Liu, T., Haber, M., Norris, M.D., Woo, A.J., Hogg, P., Wang, J., and Wang, J.Y. (2019). JMJD1C-mediated metabolic dysregulation contributes to HOXA9-dependent leukemogenesis. Leukemia *33*, 1400–1410.