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Version 2

Jun 07, 2020

UT Southwestern - Human Melanoma Metastatic Potential in Mice V.2

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1 *Works for me* This protocol is published without a DOI.

NCI PDMC consortium

Shree Bose

PROTOCOL CITATION

Arin Aurora, Sean Morrison 2020. UT Southwestern - Human Melanoma Metastatic Potential in Mice. **protocols.io** https://protocols.io/view/ut-southwestern-human-melanoma-metastatic-potentia-bg9hjz36

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CREATED

Jun 07, 2020

LAST MODIFIED

Jun 07, 2020

PROTOCOL INTEGER ID

37897

GUIDELINES

This assay is divided in 5 sections:

A.Dissociation of melanomatumors
B.Preparation of cells forinjections
C.Subcutaneous Injection of melanoma cells in NSG mice
D.Mice monitorization
E.Analysis of metastasis atendpoint.

NOTE: FACS protocol for sorting melanoma is separate

MATERIALS

NAME	CATALOG #	VENDOR
Penicillin-Streptomycin	15140122	Gibco - Thermo Fisher
PBS		
Collagenase Type 4	LS004188	Worthington Biochemical Corporation
HBSS	14025-092	Gibco - Thermo Fischer
Leibovitz's L-15 Medium	11415049	Thermo Fisher
Trypsin (2.5%), no phenol red	15090046	Thermo Fisher
40um Cell Strainer	22363547	Fisher Scientific
Deoxyribonuclease I from bovine pancreas	D4527	Sigma Aldrich
EGTA (Ethylene glycol-bis(2-aminoethylether)-NNN'N'-tetraacetic acid)	E4378	Sigma Aldrich

NAME	CATALOG #	VENDOR
PVA (Polyvinyl Alcohol)	P8136	Millipore Sigma
Bovine Serum Albumin	A3912	Sigma Aldrich
Lonza™ BioWhittaker™ Water for Cell Culture	17724Q	Fischer Scientific
Lonza™ BioWhittaker™ HEPES Buffer	BW17-737E	Fischer Scientific

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Collagenase Type 4	LS004188	Worthington Biochemical Corporation
Deoxyribonuclease I from bovine pancreas	D4527	Sigma Aldrich
Matrigel	356231	Corning
NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ	005557	Jackson Laboratory

DISSOCIATION PREPARATION

1 **Materials:**
Surgical dissection tools
Ice
1x HBSS (w/o Ca++ or Mg++) [Gibco #14175]
50 mL Falcon tubes
Disposable pipettes
40 µm cell strainers for Falcon tubes [Fisher #22363547] Cell counter

Make Stock Solutions

Collagenase Type 4
by Worthington Biochemical Corporation
Catalog #: LS004188

Collagenase Type IV: 2,000 u/mL
make up in HBSS,
Collagenase IV powder stock (Worthington #4189, u/mg varies);
freeze 1 mL aliquots at **-20 °C**

Deoxyribonuclease I from bovine pancreas
by Sigma Aldrich
Catalog #: D4527

DNase I, type II from bovine Pancreas (10,000 u/mL)
make up in HBSS from powder stock (Sigma#D4527);
freeze **0.5 mL** aliquots at **-20 °C**

$CaCl_2$ ([M]1 Molarity (M))

2 ***Make Digest Media*** (10 mL volume; make fresh every time):
9 mL HBSS
1 mL [M]**2000 units/mL** Collagenase IV Stock Solution (for final [M]**200 units/mL**)
50 µl [M]**1 Molarity (M)** $CaCl_2$ (for final [M]**5 Milimolar (mM)**)
50 µl [M]**10000 units/mL** DNase (for final [M]**50 units/mL**)

3 ***Trypsin-EGTA*** (TEG; 10x stock):
Warm **90 mL** PBS

Dissolve **40 mg** EGTA and **10 mg** PVA into warmed PBS

Add **10 mL** 2.5% trypsin (10 mL aliquots stored at –20°C) , stir and cool

pH to **pH 7.4**

Filter and store as 10 mL aliquots at –20°C

Make 1x TEG by diluting this stock in HBSS (again, freeze aliquots for later use)

4 *Staining media* (for 500ml):
500 mg BSA [Sigma #A3912]

40 mL H2O [Biowhitaker #17724Q]

5 mL **1 Molarity (M)** Hepes **pH 7.4** [Biowhittaker #17738E]

5 mL 100x PenStrep [Gibco #15140]

make up to **500 mL** with Leibovitz's L15 media

Filter and store at **4 °C**

DISSOCIATION OF MELANOMA TUMORS

5 Place tumors **On ice** in HBSS

If tumors are from mice, euthanize by anesthetizing in isoflurane followed by cervical dislocation; take care at dissection to cut off normal tissue

6 Dissect in petri dish and remove necrotic tissue (pale/whitish tissue located centrally within tumor)

7 Homogenize in homogenizing tube or chop into small pieces with disposable scalpel

8 Resuspend in 10 mL of warmed digestion media per 1g of tumor tissue (approximate)

9 Shake in 37°C water bath for 20min. Shake every **00:05:00** - **00:10:00** by hand.

10 Wash with HBSS up to 50 mL, centrifuge at **220 x g, 4°C 00:04:00**

11 Resuspend in 5 mL staining media and filter through 40 µm strainer into new 50 cc tube

From this point on keep the cells in staining medium on ice AT ALL TIMES

12 For bulk injection, proceed with next section. If sorting first, then proceed with FACS protocol located as a separate file HERE:

UT Southwestern - Staining Melanoma Cells for Flow Cytometry
by Shree Bose
PREVIEW RUN

12.1 Adjust staining volume and antibody concentration to the number of cells. Concentrations listed are for $5x10^6$ cells in a 50ml reaction. Do not exceed 10^6 cells/10ml.

- mLin APC: Ter119 (1/100), CD31(1/100), CD45(1/200)
- HLA FITC: HLA-ABC (1/5)

12.2 Add antibodies to tube and incubate covered on ice for **00:20:00**

12.3 Wash 1x with **1 mL** staining medium and spin down **220 rpm, 4°C 00:04:00** . Aspirate supernatant

12.4 DAPI staining: resuspend samples in DAPI 1:2000 to gate out dead cells. Stock in made in water (1mg/ml)

12.5 Adjust final sample volume to run at 6000 events/sec at maximum and then run. If clogging becomes an issue, then filter through cut pieces of filter

Xenograft stain (50ml volume)	1x	5x
HLA- PE or FITC (1/5)	10ml	50ml
mCD45 APC (1/200)	0.3ml	1.5ml
mCD31 APC (1/100)	0.5ml	2.5ml
mTer119 APC (1/100)	0.5ml	2.5ml

Human stain (50ml volume)	**1x**	**5x**
HLA- FITC (1/5)	10ml	50ml
ISO PE or P75 PE (1/5)	10ml	50ml
Glyc-A APC (1/2000)	0.025ml	0.125ml
hu-CD31 APC (1/800)	0.063ml	0.315ml
hu-CD45 APC (1/5)	10ml	50ml

PREPARATION OF CELLS FOR INJECTION PREPARATION

13 **Materials**
Ice
Eppendorfs
500cc insulin syringes BD #329461 Hemocytometer
Trypan Blue

Stock solutions:

Matrigel
by Corning
Catalog #: 356231

Thaw overnight in ice, keep **On ice** at all times

Add **10 mL** of cold staining media to bottle

Mix by gently inverting for **00:05:00** aliquot and store at **-20 °C**

PREPARATION OF CELLS FOR INJECTION

14 Prepare 10 empty sterile syringes on a Styrofoam box filled up with ice.

You will need to thaw the right amount of 50% matrigel 2h before preparing cells. Matrigel should be kept on ice at ALL TIMES to be thawed.

15 Centrifuge sorting tube containing cells at **220 x g, 4°C 00:04:00**

16 Resuspend pelleted cells in a small volume that will allow accurate counting.

17 Count cells in hemocytometer with Trypan blue to determine concentration of cells cells.

If sorted, may lose roughly 20-30%

18 For 10 injections, prepare **700 µl** of cells at 100 cells/50 uL in 25% matrigel.

18.1 Prepare a cell suspension of 1400 sorted cells into **350 µl** of cold SM.

18.2 Add to above **350 µl** of 50% matrigel solution and resuspend with pipet up and down 5 times to get to an even solution of cells.

19 Load 10 syringes with **50 µl** of the cell suspension each and maintain loaded syringes on ice at all times.

Take leftover cells on ice in case you need extra in mouse room

INJECTION PREPARATAION

20 **Materials:**
Syringes loAded with 50 l of sorted melanoma cells in matrigel
50 ml conical tube with holes in tip to allow mouse ventilation.
Mice injection sheet with records (see example attached)

NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ
by Jackson Laboratory
Catalog #: 005557

SUBCUTANEOUS INJECTION OF MELANOMA CELLS IN NSG MICE

21 Choose a cage with 5 mice (at least 4 weeks old) and write down information on mice injection sheet (cage ID, gender and date of birth)

22 Write your name, the injected tumor ID, date and “metastasis assay” on the cage card.

23 Prepare a clean cage where you will transfer mouse by mouse as soon as the injection is done.

24 Choose one mouse from uninjected cage, immobilize mouse and ear tag (see guide).

25 Restrain mouse in the conical 50ml tube and inject slowly subcutaneously in right flank. After injecting press injected site with your fingers for a few seconds.

26 Transfer mouse to the clean cage and proceed with the next mouse.

MICE MONITORIZATION

27 Mice will be monitored every 15 days by palpation at site of injection

Once tumors become measurable, measure and write down the biggest diameter weekly