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We use this protocol and it's working

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Preparation and cryopreservation of human liver samples for analysis by flow cytometry (fresh or after cryobanking)

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ABSTRACT

This protocol focuses on the preparation of small **human liver samples** for single-cell analysis by flow cytometry. It provides two options for sample preparation: (1) immediate flow cytometry with **fresh** samples or (2) **cryopreservation** of samples at -80°C using the **Stable-Lyse/Store V2** system from SmartTube Inc. for flow cytometry at a later timepoint.

Note: This protocol does not provide guidelines on how to create a multicolor flow cytometry panel!

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Keywords: human samples, liver, liver tumor, HCC, Flow Cytometry, Banking, FACS, human, immune monitoring, immune microenvironment

GUIDELINES

1. This protocol is validated for **immune cell isolation** of **human liver and tumor specimen** (weight 150-1000 mg). It has not yet been tested on biopsy material (< 20 mg).
2. The retrieval of **parenchymal cells** is a by-product of this protocol but it has not been optimized for this purpose!
3. Experiments should be performed **on ice** at all times!
4. Work with **filter tips**!
5. When working with **fluorochrome-conjugated antibodies**, avoid bright (sun) light and consider using aluminum foil to protect your samples from light while incubating.
6. For **waste management and time reasons**, transfer of e.g. larger liquid volumes is done by pouring it directly from the bottle. If you prefer otherwise, you can always use serological pipets.

Advantages:

1. Isolation of all major immune cell subtypes including **granulocytes.**
2. **Nycodenz gradient** ensures minimal amount of parenchymal cells which improves the quality of the FACS analysis.
3. **Ready-to-use** quality-controlled cryo-preservation buffers.

Limitations:

1. As mentioned above, this protocol has **not been tested** on **biopsies** (< 20 mg tissue).
2. If you also focus on **isolation of hepatocytes**, two-step-collagenase perfusion of bigger liver specimen might be a more suitable approach.

Qualifications:

We recommend **basic experience in wet-lab work** (e.g. how to pipette) to handle this protocol. You also might need a trial run to familiarize yourself with the procedures.

MATERIALS

INSTRUCTIONS FOR STOCK AND BUFFER PREPARATIONS:

- 25% BSA aliquots
- Blocking Buffer
- GBSS
- Stop Digest Buffer
- HBSS + 0,1% BSA
- FACS Buffer
- Fixaton Buffer
- 1X Lysis Buffer

Buffers for Human Liver and Tumor Immune Cell Isolation.pdf

REAGENTS:

- Albumin bovine-serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4503**
- Calcium chloride dihydrate ≥99 % p.a. ACS **Carl Roth Catalog #5239.2**
- Collagenase B (Roche) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11088815001**
- D( )-Glucose p.a. ACS anhydrous **Carl Roth Catalog #X997.2**
- di-Sodium hydrogen phosphate dihydrate ≥990 % p.a. **Carl Roth Catalog #4984.2**
- DNase I (Roche) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10104159001**
- 1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific Catalog #14190094**
- EDTA - Solution pH 8.0 **Panreac AppliChem Catalog #A3145**
- Gibco™ HBSS without Calcium without Magnesium ohne Phenolrot **Thermo Fisher Scientific Catalog #14175053**
- Gibco™ RPMI 1640 Medium (with L-Glutamine) **Thermo Fisher Scientific Catalog #21875034**
- 4% Paraformaldehyde (PFA) Solution in PBS **Boster Bio Catalog #AR1068**
- Nycodenz AG® **Proteogenix Catalog #1002424**
- Magnesium chloride hexahydrate ≥99 % p.a. ACS **Carl Roth Catalog #2189.1**
- Magnesium sulphate heptahydrate ≥99 % p.a. ACS **Carl Roth Catalog #P027.1**
- Potassium chloride ≥995 % p.a. ACS ISO **Carl Roth Catalog #6781.3**
- Potassium dihydrogen phosphate ≥99 % p.a. ACS **Carl Roth Catalog #3904.1**
- BD Pharm Lyse™ **BD Biosciences Catalog #555899**

Sodium hydrogen carbonate ≥995 % p.a. ACS ISO **Carl Roth Catalog #6885.2**

REAGENTS ONLY NEEDED FOR CRYOFIXATION:

Stable-Lyse V2 **SMART TUBE Inc. Catalog #Stable-Lyse V2**

Stable-Store V2 **SMART TUBE Inc. Catalog #Stable-Store V2**

REAGENTS ONLY NEEDED FOR FLOW CYTOMETRY:

Sera from human **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S2257-1ml**

Sera from mouse **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S3509-1ml**

Normal rabbit serum (invitrogen) **Thermo Fisher Scientific Catalog #10510**

Normal rabbit serum (invitrogen) **Thermo Fisher Scientific Catalog #10710C**

+ **fluorochrome-conjugated antibodies of your choice**
+ **fixable viability stain** (if desired)

DISPOSABLES OF YOUR CHOICE:

- Cell Strainer 100 µM (for 50 ml Falcon tubes)
- Centrifuge tubes (15 ml, 50 ml)
- Cryotubes (1,8 ml)
- Microcentrifuge tubes (1,5 ml, 2 ml)
- Needles (20G)
- Petri dishs (6 cm)
- Pipet filter tips (10, 200 and 1000 µl)
- Scalpel
- Serological pipets (5 ml, 10 ml, 25 ml)
- Syringes (10 ml)
- Urine cups (100 ml)
- 5 ml FACS tubes with 35 µM cell strainer cap
- caps for FACS tubes

EQUIPMENT:

- biosafety cabinet
- centrifuge

- (shaking) waterbath
- ice buckets
- small scissors
- fine scale
- small forceps
- pipets (0,1-2,5 µl, 0.5-10 µl, 10-100 µl, 100-1000 µl)
- pipette controler

SAFETY WARNINGS

If your work with **untested human samples** (e.g. status of Hepatitis B/C and HIV is unknown), all experiments must be carried out in a **Class II biosafety cabinet (with laminar air flow)**. Liquid waste has to be collected and **autoclaved** before disposal, contaminated materials have to be collected and **disposed of separately** from other lab waste as they are potentially infectious.

Use **caps/lids** to close your **FACS tube** when removing them from the biosafety cabinet for centrifugation. Use pipet tips with **filters**.

Depending on your personal safety preferences as well as the specific regulations of your research department/institute/country, there might be other or additional regulations to consider. Contact your **biosafety officer**!

ETHICS STATEMENT

Before you work with human samples, you have to **acquire an human ethics approval** from the local ethics committee of your institution! Every subject/patient has to give **informed consent**!

BEFORE START INSTRUCTIONS

1. Make sure you read the safety warnings regarding **untested human samples**!
2. Make sure an **ethics approval** has been obtained before working with subjects or patient samples!
3. Revisit the **materials list** to make sure all the equipment, materials and reagents are available to you.

Preparations (before samples are aquired from the OR)

30m

1 Start the biosafety cabinet. 1m

2 Prepare all the **reagents and equipment** needed for cell isolation.

2.1 Prechill a **centrifuge** to 4 °C as well as preheat a (shaking) **waterbath** to 37 °C . 2m
Place a 50 ml falcon tube containing **HBSS** inside the waterbath to warm up.

2.2 Place all needed **buffers and reagents**, including a 50 ml falcon tube containing **HBSS**, 3m
On ice or in the wasterbath.

Note

- 2x HBSS in 50 ml Falcon (4°C and 37 °C)
- Stop Digest Buffer (on ice)
- HBSS +0.1% BSA
- 1x Lysis Buffer
- FACS buffer

(when you start FACS right after isolation)

- Blocking Buffer

2.3 Place **urin cups filled with around 50 ml Medium** (RPMI) for your samples On ice . 3m

2.4 Freshly weigh **Collagenase B** (**8.52 mg** per sample) and **DNase I** (**1.875 mg** per sample) in **2** 10m
ml tubes and place them On ice until further use.

Note

You may prefer to predilute your enzymes and freeze them in **aliquots**. We choose to prepare the enzymes freshly for **stable enzyme activity**.

2.5 Prepare **Nycodenz stock solution** (**14ml** per sample) and place On ice .

Note

- per sample weigh 4g of Nycodenz in a 50 ml Falcon
- add 14 ml of GBSS to the Falcon
- close the top tightly and vortex until half of the Nycodenz has dissolved
- let the falcon rest on the side on the countertop for 5 min
- vortex again until dissolved completely
- keep on ice

2.6 Set up the **biosafety cabinet** with all the equipment you'll need and make sure, everything you will need can be reached easily. 5m

Note

Preparing all the equipment you need will save you ample time. **Working fast** is key in recovering viable liver/tumor immune cells.

3 Directly before **leaving for the OR to** pick up the samples, place the **Collagenase** in the waterbath to preheat. 1m

Digestion (BSL2) 50m

4 Place the **liver samples** in the urine cups with medium, and transport them back to the lab On ice . Place samples (still on ice) inside the biosafety cabinet.

Note

CAVE - With the exception of the digestion, which takes place at **37 °C**, place your samples **on ice** at all times!

5 Dissolve the **DNase I** in an appropriate amount of **cold HBSS** (7.5 mL per sample) and keep On ice in a 15 ml Falcon. 3m

Note

HOW TO - Using a 1000 µl pipette, dissolve the DNase in 1 ml cold HBSS and transfer to a 15 ml falcon tube. Add remaining amoutn of cold HBSS and mix well.

6 Dissolve **Collagenase B** in an appropriate amount of **37 C° warm HBSS** (3 mL per sample) in a 15 ml Falcon. Transfer back to 37 °C until needed for digestion. 3m

7 **Weigh the tissue samples** and place in **petri dishes** on a cooled sample holder. 5m

8 * If your samples are very bloody, you may attempt to **flush** the samples. For this, fill a **10 ml syringe** with **cold HBSS** and attach a **needle**. **Puncture the sample** and carefully press the HBSS into the tissue to flush out the blood. **Repeat** this process several times until the liquid runs mostly clear. Discard the liquid before proceeding. 5m

9 **Cut sample** into very small pieces using first a **scapel** and then **fine scissors** until the whole tissue appears **pulpous**. 3m

10 Add 3 mL **Collagenase B** to each petri dish, **swirl** to mix and transfer to a **50 ml falcon** by pouring the liquid directly into the tube. Use a small forceps to get all the tissue pieces out of the petri dish. 2m

11 Add 250 µL **DNase I** to each falcon and close the top tightly. Mix by swirling the falcon 1m

12 Immediately transfer to a **shaking waterbath** (37 °C , 150 rpm) and digest for 00:30:00 . **Shake the sample vigorously** every 5 min! 30m

Note

CAVE - If you do not have a shaking waterbath, any waterbath will do. However, it is **crucial** to shake the sample on a regular basis!

13 After digestion, place falcons On ice and immediately add 10 mL **cold Digest Stop** to end the digestion process. 2m

Filtering and Lysis (BSL2) 1h 42m

14 Use a **10 ml serological pipet** to pipette the cell suspension up and down multiple times to further detach the cells from their connective tissue. When the cell suspension **runs easily** through the pipet, proceed to the next step. 2m

15 Using the same 10 ml serological pipet, filter the sample through a **100 µm cell strainer** into a new 50 ml falcon. 1m

16 Use 20 mL **cold Digest Stop** to rinse the old falcon. Gently **press** the remaining tissue through the cell strainer with the smooth end of a **syringe plunger**, repeatingly rinsing the mesh with the Digest Stop from the old falcon. 5m

Note

CAVE - Press the plunger straight down on the cell strainer and release in tapping motions.

17 Remove the cell strainer and add 3 mL **DNase I** to the cell suspension.

18 Spin 500 x g, 4°C, 00:05:00 . 5m

19 Place your falcons back On ice and **discard the supernatant** by pouring it into the liquid waste bottle without disrupting the pellet. 1m

20 If you want to perform **lysis** right away, continue here. Otherwise, continue with **step 21**.

Note

GOOD TO KNOW - The gradient will also eliminate erythrocytes - if you do not plan to work with the parenchymal cells, you can skip this step. You can also treat parenchymal cells with lysis buffer at a later timepoint or use an erythrocyte depletion kit.

20.1 Resuspend the cells by gently flicking the tube or pipetting up and down. Add 2 mL **1X Lysis Buffer** , gently pipette up and down to mix and incubate for 00:05:00 On ice . 5m

20.2 Add 40 mL **cold Digest Stop** and 4 mL **DNase I** to each falcon. 2m

21 Spin 50 x g, 4°C, 00:02:00 to pellet the parenchymal cells. 2m

Note

If you set focus on isolating **parenchymal cells** (hepatocytes), you might extent this step to 00:05:00 - keep in mind that you might lose some of your non-parenchymal cells when you do so.

Furthermore, fractions of parenchymal cells will be relatively small, as the tissue section is cut and digested without perfusion.

22 Transfer the supernatant to a new falcon and spin 500 x g, 4°C, 00:10:00 . 10m

23 Discard the supernatant and add **cold HBSS + 0,1% BSA** with a serological pipet to a final volume of 7.4 mL and gently resuspend. 2m

24 Add 12.6 mL **Nycodenz stock solution** with a serological pipet and gently mix until homogenous. 1m

25 **Overlay** the Nycodenz-Cell Suspension with 6 mL **HBSS + 0,1% BSA.** 5m

Note

HOW TO OVERLAY - Due to the higher density of Nycodenz, HBSS will layer on top if it is applied carefully.

1. The **dispension force** of your pipette controller has to be deactived.
2. Hold the 50 ml **falcon** containing the Nycodenz-cell suspension at a **45° angle**.
3. Aspirate 6 ml of HBSS into a **10 ml serological pipet** and **press the tip to the tube wall** in a 90° angle (the tip opening should be flat on the tube wall).
4. Very **slightly press the dispense button** and let the HBSS run down the side of the tube in a **constant trickle** without any drops forming.

26 Transfer the falcon tubes to a centrifuge and spin 1400 x g, 4°C, 00:22:00 ***without break*** (takes around 45min total). 35m

27 After centrifugation, place the tubes back On ice . Carefully remove the first 2-3 ml from the top layer (debris and dead cells) and discard. Next, use a **10 ml serological pipet** to slowly **remove the white interphase** (middle layer containing the **immune cells**) and transfer to a new falcon tube. 5m

Note

You can also use a sterile **pasteur pipet** to remove the middle layer.

28 Add **cold HBSS + 0,1% BSA** to a **final volume** of 30 mL and spin 500 x g, 4°C, 00:10:00 . 10m

29 Discard the supernatant without disrupting the pellet and resuspend the cells in 2 mL **cold FACS buffer**. 1m

30 Pass the cell suspension through a **35 µm cell strainer cap into FACS tubes** placed On ice . Discard the cell strainer cap afterwards. 2m

Safety information

Always close the FACS tubes **with a cap** before taking them out of the biosafety cabinet!

31 Spin 500 x g, 4°C, 00:05:00 5m

32 **Discard the supernatant** and resuspend the cell pellet in the remaining buffer by flicking the tube. 1m

Note

HOW TO DECANT - Empty the supernatant with momentum and - while keeping the FACS tube in an upside-down position to not disrupt the pellet on the bottom - dip the tube onto a paper towel to get rid of the excess liquid. Turn the FACS tube upright and gently flick to resuspend.

Choose protocol according to desired further sample proces..

33 Depending on whether you want to perform flow cytometry on fresh or cryo-preserved samples, you can select different protocol options at this step.
Step 33 includes a Step case.
FACS fresh
FACS later

Preparations for Stainings (BSL2)

step case

FACS fresh

Use this protocol if you want to perform flow cytometry immediately after immune cell isolation.

34 Label your **5 ml FACS tubes** and place them in a cold sample holder or On ice . You might need several tubes per sample, depending on the number of panels and controls you are planning to run.

Note

GOOD TO KNOW - As liver samples are very autofluorescent, recording an unstained control for every sample will give you a far better unmixing/compensation result.

FMO (fluorescence minus one) controls are helpful to discriminate positive and negative (unspecific background fluorescence) signals, especially if the positive cell populations are not distinctly separated from the negative population. In an FMO tube, all fluorochromes in the panel are present except the fluorochrome in question.

35 Prepare the antibody mix: For each FACS tube, use 20 µL **blocking buffer** and the appropriate amount of each of your **antibodies**. Always prepare the master mix for one extra sample or add 10% to account for pipetting errors.

Note

EXAMPLE 1

- 2 samples (one full stain and one control each) + 1 extra for pipetting error = 5x mix
- 20 antibodies in panel (1µl needed per sample)
- Blocking Buffer: 5 x 20 µl = 100 µl
- 5 µl per antibody
- final volume of antibody master mix: 200 µl
- mix to add per FACS tube = 20 µl blocking buffer + 20 µl antibodies = 40 µl

EXAMPLE 2

- 2 samples (one full stain and one control each) = 4x mix +10%
- 20 antibodies in panel (1µl needed per sample)
- Blocking Buffer: 4x 20 µl = 80 µl (+ 10%) = 88 µl
- Each antibody: 4 µl per antibody +10% = 4,4 µl per antibody
- final volume of antibody master mix: 176 µl
- mix to add per FACS tube = 20 µl blocking buffer + 20 µl antibodies = 40 µl

36 **Divide** the cell suspension between the different FACS tubes. Its important to **document** the amount of sample going into each tube to allow determination of absolute cell numbers during analysis (eg., calculate **cells/ gram tissue**).

37 Bring the volume in each FACS tube to 100 µL with **FACS buffer**. Set aside the **unstained control.**

Staining (BSL2)

5m

38 If you prefer, switch of the light in the biosafety cabinet/lamina flow hood to avoid bleaching the fluorochrome-conjugated antibodies.

39 Add the appropriate concentration of **fixable viability stain** to the **cell suspension** in each of the FACS tubes and mix well by flicking the tube.

Note

HOW TO "FLICK"- Mix well by holding the upper part of the tube between thumb and index finger of one hand and at the same time, gently flick the bottom of the tube repeatedly with the index finger of the other hand.

40 Incubate for 00:05:00 On ice . 5m

41 Add the appropriate amount of the **prepared antibody mix** to each tube. Gently pipette up and down to mix.

Note

EXPERT TIP - In case you are using FMO control(s), you can prepare the antibody mix for all your tubes without the FMO antibody(ies) and add them later to your full stain tube.

42 Incubate for 00:20:00 On ice . 20m

Washing and Fixation (BSL2) 20m

43 Add 2 mL **FACS buffer** and mix well by pipetting up and down.

44 Spin 500 x g, 4°C, 00:05:00 5m

45 **Discard the supernatant by decanting** and resuspend the cell pellet in the remaining buffer by flicking the tube.

46 Add 1 mL **Fixation Buffer** and mix well by pipetting up and down.

47 Incubate for 00:10:00 at On ice . 10m

48 Add 2 mL **FACS buffer** and mix well by pipetting up and down.

Note

After fixation, samples can be further processed on the bench, a biosafety cabinet is no longer necessary.

49 Spin 500 x g, 4°C, 00:05:00 5m

50 **Discard the supernatant** and resuspend the cell pellet in the remaining buffer by flicking the tube.

51 Add 200 µL **FACS buffer** and proceed to sample aquisition.

Note

Fixed samples can be stored at 4 °C overnight and recorded the next day. However, keep in mind that fixatives such as formalin can change the autofluorescence of your cells and destabilize fluorochromes, especially tandem dyes. If possible, record you samples directly after fixation.