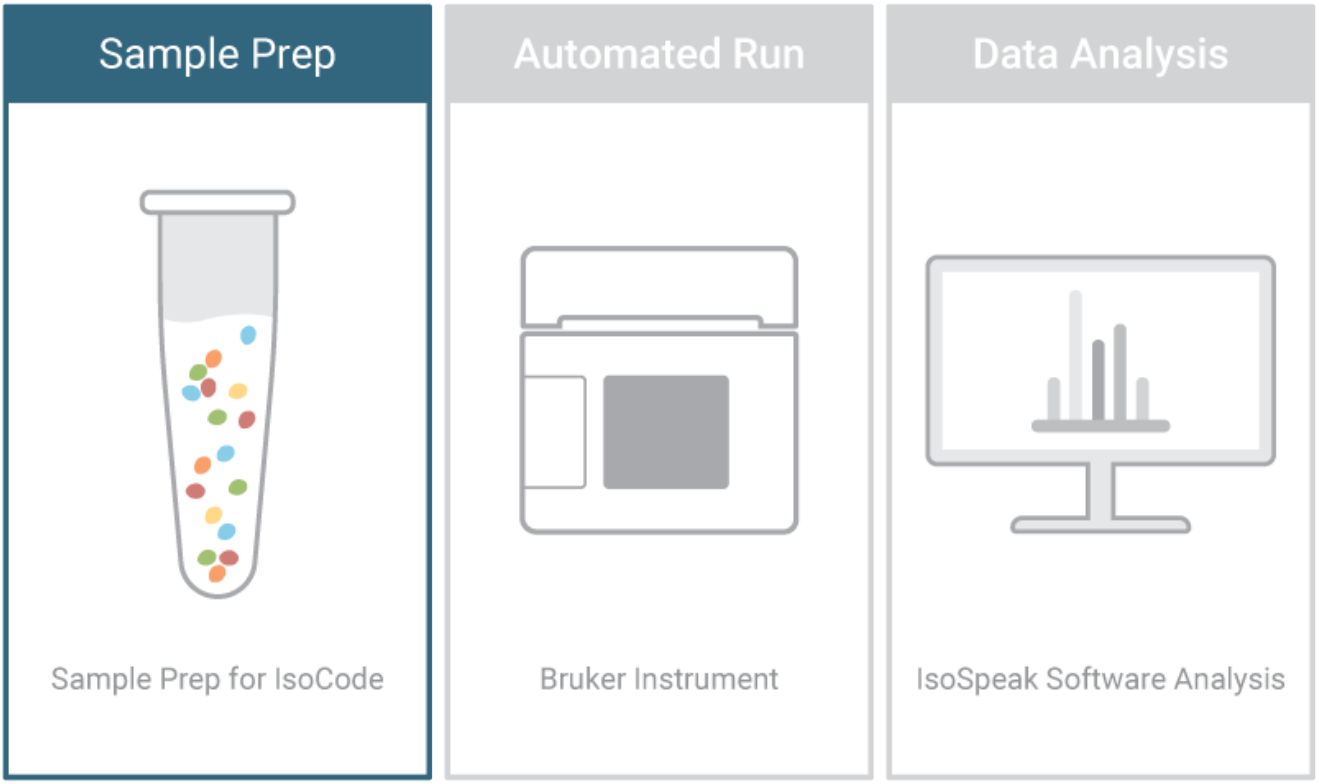
IsoCode Single-Cell Adaptive Immune: Human CAR-T Protocol Using AF647-Conjugated Antibodies to CD4 and CD8

Ensure you achieve the maximum benefit from the Bruker systems and generate impactful data as quickly as possible



Key: ● TIP, ● CRITICAL, ● OPTIONAL

Contents

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A. Overview

Overview of Protocol

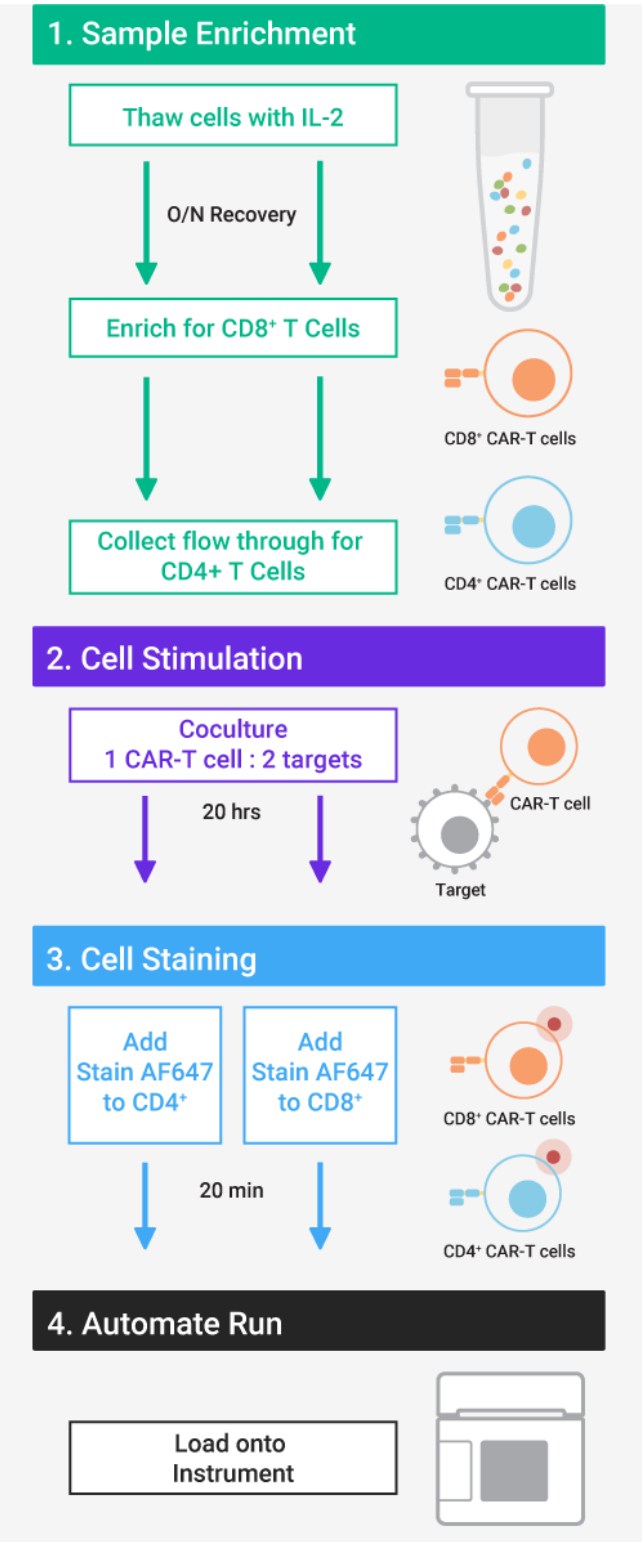
Day 1: Cryopreserved CAR-T cells are thawed and cultured overnight in the presence of IL-2. Cryopreserved target cell lines are thawed, cultured, and passaged several days prior to beginning the experiment.

Day 2: Enrichment and Antigen-Stimulation of CD8+ and/or CD4+ CAR-T cells for 20 hours.

Day 3: Staining and Loading of CAR-T cells onto IsoCode chip.

NOTE:
This protocol outlines the standard method for thawing and culturing of human CAR-T cells only and may not be valid for other species or cell types.

NOTE:
Using stains and protocols other than the included kit surface stains and protocols might result in failed runs. Stains and staining procedures not approved by Bruker will require validation prior to use. Please consider Bruker's IsoPACE program to assist in custom marker and protocol validation.



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PRO-9 REV 7.0

Safety Warnings

- Read MSDS documents of all materials prior to use.

- Laboratory workers should wear standard PPE, including disposable gloves, protective eyewear, and laboratory coats.

Required Reagents, Consumables, and Equipment

Table 1: Required Consumables Provided by Bruker

Item	Catalog Number	Quantity	Comment
IsoCode Kit	Please see website (https://brukercellularanalysis.com/) for available kits or talk to Bruker's Customer Service team for details	One chip per sample/cell type/condition	Shipped at 4°C and -20°C; subcomponents stored at 4°C and -20°C

IsoCode Kit Components

IsoLight IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- 50 mL Tubes containing Reagents 1, 2, 3, 4, 5, 6, 7, 8
- 1 Bag of Disposable Reagent Sippers
- Alexa Fluor 647 anti-human CD4 stain (AF647-CD4) [ordered separately]
- Alexa Fluor 647 anti-human CD8 stain (AF647-CD8) [ordered separately]

IsoSpark IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- Cartridge containing Reagents 1, 2, 3, and 4
- Alexa Fluor 647 anti-human CD4 stain (AF647-CD4) [ordered separately]
- Alexa Fluor 647 anti-human CD8 stain (AF647-CD8) [ordered separately]

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 per box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits

Table 2: Required Consumables Not Supplied by Bruker

Consumable	Type	Source	Catalog Number
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
6 Well Plate Flat Bottom	N/A	Corning	353046
24 Well Plate Flat Bottom	N/A	Corning	3524
48 Well Plate Flat Bottom	N/A	Corning	3548
96 Well Plate U-Bottom	N/A	Corning	353077
MACS LS Column	N/A	Miltenyi	130-042-401
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	62406-200
Lo-Bind Microcentrifuge Tubes, Sterile	1.5 mL	USA Scientific	4043-1081
Pipette Tips (Filtered)	10 µL Graduated Filter Tips 100 µL Graduated Filter Tips 1000 µL XL Graduated Filter Tips	USA Scientific	1181-3710 1183-1740 1182-1730
Serological Pipette	2 mL Pipette 5 mL Pipette 10 mL Pipette	USA Scientific	1072-0510 1075-0110 1071-0810
Syringe with BD Luer-Lok Tip	10 mL	VWR	75846-756
0.2 µm Syringe Filter with Acrylic Housing	N/A	VWR	28145-501
Fisherbrand Disposable PES Filter Units (0.20 µm)	500 mL	Fisher Scientific	FB12566504
Polystyrene Round Bottom Tube	5 mL	Falcon	352058

*Bruker strongly recommends that low protein binding centrifuge tubes are used for cell culture work to ensure optimal cell pelleting.

Table 3: Required* Reagents Not Supplied by Bruker

Reagent	Stock Concentration	Source	Catalog Number
RPMI	1x	Fisher	MT10040CV
Penicillin-Streptomycin-Neomycin Solution Stabilized	100x	Sigma	P4083-100mL
Glutamax	100x	Thermo	35050061
FBS	1x	Sigma	F2442-6X500mL
Recombinant IL-2	200 µg/mL	Biolegend	589104
Bovine Serum Albumin (BSA), lyophilized powder	N/A	Sigma-Aldrich	A9647-10G
Phosphate Buffered Saline (1X PBS) without Calcium or Magnesium	1x	Gibco	10010072
RoboSep buffer	1x	StemCell Tech	20104
Ficoll Paque Plus	N/A	GE Healthcare	17-1440-03
Miltenyi CD8 Microbeads, Human, 2mL	N/A	Miltenyi	130-045-201
Miltenyi CD4 Microbeads, Human, 2 mL (if needed)	N/A	Miltenyi	130-045-101
TrypLE Express Enzyme (1X), no phenol red	1x	Gibco	12604021
Trypan Blue	0.4%	Gibco	15250-061
Negative control target cells		Target-type dependent	
CAR-T specific target cells		Target-type dependent	
Reagent Alcohol 70%	N/A	Lab Grade	N/A
Dynabeads M-280 Streptavidin	N/A	Thermo-Fisher	11205D (2 mL)
Biotin anti-human CD235a clone: HIR2 (GA-R2) – K562 Cell Line	0.5 mg/ml	Invitrogen	13-9987-82
Biotin eBioscience anti-human CD19 Monoclonal Antibody clone: HIB19 – Raji Cell Line	0.5 mg/ml	Invitrogen/Thermo-Fisher Scientific	13-0199-82

*Reagents have been validated by Bruker and no alternatives may be used.

Table 4: Cell Staining Reagents

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Test Material	Catalog Number	Color
Alexa Fluor 647 anti-human CD8 (AF647-CD8)	STAIN-1002-1	Red
Alexa Fluor 647 anti-human CD4 (AF647-CD4)	STAIN-1003-1	Red

Table 5: Required Equipment

Equipment	Source	Catalog Number
IsoLight, IsoSpark, or IsoSpark Duo Instrument	Bruker	ISOLIGHT-1000-1, ISOSPARK-1000-1, or ISOSPARK-1001-1
Culture Hemocytometer	(Fisher) Hauser Levy	02-671-55A
Hemocytometer Cover Glass	(Fisher) Hauser Levy	02-671-53
MidiMACS Separator	Miltenyi	130-042-302
MACS MultiStand	Miltenyi	130-042-303
EasySep Magnet	StemCell Technologies	18000

Table 6: General Equipment

Equipment	Requirements
Pipette	P10, P100, P200, P1000
Pipettor	Ability to pipette between 1 and 10 mL
Incubator	37°C, 5% CO_2
Tabletop Centrifuge	Temperature controlled*; swinging bucket rotor; ability to centrifuge 15mL conical tubes
Microcentrifuge	Temperature controlled*; fixed rotor; ability to centrifuge 1.5 mL microcentrifuge tubes
Mini centrifuge	Ability to spin micro sample sizes
Water Bath	Ability to heat to 37°C
Microscope	Inverted light microscope with 10x and 20x objectives
Vortex Mixer	Ability to vortex vials and microcentrifuge tubes; adjustable speed

*Temperature controlled centrifuges are required so that centrifuging steps can be conducted at room temperature without risk of overheating. Temperature on centrifuges should be set to 21°C.

B. Before Getting Started

1. Important Precautions

Read MSDS documents of all materials prior to use.

Working with Biohazardous Reagents

Please refer to your institute's guidelines and obtain proper training to handle potentially biohazardous samples. It is also strongly recommended that any lab personnel handling human samples should be vaccinated against HBV if the individual does not have sufficient HBV antibody titer.

Additional precautions need to be taken when working with samples that potentially contain an EID agent:

1. Laboratory workers should wear standard PPE, including disposable gloves, protective eyewear, and laboratory coats.
2. Any procedure or process that cannot be conducted in the designated EID BSC should be performed while wearing gloves, gown, goggles, and a fit tested N-95 mask.
3. Work surfaces should be decontaminated on completion of work with appropriate disinfectants. This includes any surface that potentially comes in contact with the specimen (centrifuge, microscope, etc.).
4. All liquid waste produced in the processes must be treated to a final concentration of 10% bleach prior to disposal.

2. Reagents to be Prepared Before Starting

Table 7: Complete RPMI Recipe

- CRITICAL: Complete RPMI media has been validated for use by Bruker. Using alternative media may result in failed runs. Please contact your Field Application Scientist for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 500 mL	Vendor/Catalog
Penicillin-Streptomycin-Neomycin Solution Stabilized	100x	1x	5 mL	Sigma/P4083-100mL
Glutamax	100x	1x	5 mL	Thermo/35050061
FBS	100%	10%	50 mL	Sigma/F2442-6X500 mL
RPMI	1x	1x	440 mL	Fisher/MT10040CV

Note | Sterile-filter through 0.20 µm filter before use. Store complete RPMI Media at 4°C and warm up to 37°C in water bath prior to use.

Table 8: 1% BSA Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 100 mL	Vendor/Catalog
Bovine Serum Albumin (BSA), lyophilized powder	N/A	1%	1 g	Sigma-Aldrich/ A9647-10G
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1X	1X	99 mL initially*	Gibco/10010072

*Rotate solution until BSA powder is dissolved and then bring final volume up to 100 mL with 1X PBS.

Table 9: 0.1% BSA Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 100 mL	Vendor/Catalog
Bovine Serum Albumin (BSA), lyophilized powder	N/A	0.1%	0.1 g	Sigma-Aldrich/ A9647-10G
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1X	1X	99.9 mL initially*	Gibco/10010072

*Rotate solution until BSA powder is dissolved and then bring final volume up to 100 mL with 1X PBS.

Table 10: Working Stock of Recombinant IL-2 (1 µg/mL) Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 10 mL	Vendor/Catalog
1% BSA in 1X PBS (sterile filtered)	1%	1%	9.95 mL	Table 8
Recombinant IL-2	200 µg/mL	1 µg/mL	50 µL	BioLegend/589104

Note | Sterile-filter through a 0.2 µm PES filter before use.

- CRITICAL: Prepare 200 µL IL-2 aliquots and freeze at -20°C for no longer than 1 month. Aliquots are single use only and are to be thawed immediately prior to their usage. If there is any remaining volume in an aliquot, do not refreeze but discard.

Bulk Depletion Bead Preparation

- CRITICAL: Prepare target cell line depletion beads prior to starting this protocol when utilizing suspension target cells. See Appendix D3 for instructions. Appendix D3 describes the protocol for how to conjugate depletion beads for two widely used target cells; K562 cells (CD235a) and Raji cells (CD19). These depletion beads can be used for any target cells expressing CD235a or CD19 respectively.

C. Protocol

Chapter 1: Getting Started

Kit Contents

IsoLight IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 15 mL Tube A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- 50 mL Tubes Containing Reagents 1, 2, 3, 4, 5, 6, 7, 8
- 1 Bag of Disposable Reagent Sippers
- Alexa Fluor 647 anti-human CD8 stain (**AF647-CD8**) [ordered separately]
- Alexa Fluor 647 anti-human CD4 stain (**AF647-CD4**) [ordered separately]

IsoSpark IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- Cartridge containing Reagents 1, 2, 3, and 4
- Alexa Fluor 647 anti-human CD4 stain (**AF647-CD4**) [ordered separately]
- Alexa Fluor 647 anti-human CD8 stain (**AF647-CD8**) [ordered separately]

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 Per Box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits

Chapter 2: Recovery of Cryopreserved Target Cell Lines

Materials Required

Complete RPMI (37°C)
15 mL Centrifuge Tube (*Target Cells*)
Cryopreserved Target Cell Lines
Plate and/or Flask
For > 10 M Cells, T75 Flask
For 6-9.9 M Cells, T25 Flask
For < 6 M, 6 Well Plate

All the following steps should take place in a sterile tissue culture hood.

Methods

- CRITICAL: Prepare target cell line depletion beads prior to starting this protocol when utilizing suspension target cells. See Appendix D3 for instructions. Appendix D3 describes the protocol for how to conjugate depletion beads for two widely used target cells; K562 cells (CD235a) and Raji cells (CD19). These depletion beads can be used for any target cells expressing CD235a or CD19 respectively.
- CRITICAL: This chapter should be performed at least three days prior to the recovery of CAR-T cells (Chapter 4). *A week in advance is recommended.*

1. Pipette 5 mL of complete RPMI into a 15 mL centrifuge tube, labeled *Target Cells*.
2. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. TIP: Be careful of contamination.
3. Quickly move vial(s) into a water bath (37°C) to thaw. While thawing, swirl the vial in the water until a single ice crystal remains in the vial. Be sure to prevent (to the best of your ability) any of the water from the water bath from getting under the cap and into the sample.
4. When the sample is nearly thawed, remove the vial and immediately spray vial with 70% alcohol before bringing into the hood. It is important to allow the alcohol to evaporate before opening the vial.
5. Slowly pipette thawed cells into the 5 mL of complete RPMI in 15 mL centrifuge tube, labeled *Target Cells*. TIP: Insert tip into complete RPMI when pipetting, be careful to not create bubbles.
6. Take 1 mL of complete RPMI and pipette into original thawed cell vial. Rinse inside the vial with the complete RPMI to recover additional thawed cells. TIP: Insert tip into complete RPMI, be careful not to create bubbles.
7. Draw up cell/complete RPMI mixture and pipette into the 15 mL centrifuge tube, labeled *Target Cells*. TIP: Insert tip into complete RPMI and pipette gently up and down. Be careful not to create bubbles.
8. Centrifuge cells for 10 minutes at 300 rcf.
9. Remove cells from centrifuge, check for cell pellet.
10. Aspirate supernatant. TIP: Be careful not to aspirate the cell pellet.
    a. Use pipette to remove last bit of supernatant.
11. Resuspend cell pellet in 1 mL of fresh complete RPMI. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
12. Slowly add complete RPMI to a final concentration of 1 x 10^6 cells/mL.
13. Mix well 5 times with serological pipette. TIP: Be careful not to create bubbles.
14. Transfer cell suspension to flask or plate. TIP: Slowly pipette down the side of the flask as to not create bubbles.
15. Spread out cell suspension by rocking flask or plate carefully to fully cover the bottom of the container. TIP: Be careful to not make bubbles.
16. Incubate at 37°C, 5% CO_2.
17. Passage cells every few days, depending on the requirements for specific cell type.

Chapter 3: Culture of Target Cells

NOTE: Please read before proceeding with culture of target cells.

There are 2 sections depending on your type of target cell:

1. Culture of suspension target cells as described in Chapter 3a.
2. Culture of adherent target cells as described in Chapter 3b.

Chapter 3a: Culture of Suspension Target Cells

Materials Required

Complete RPMI (37°C)
Incubated Cells from Chapter 2
15 mL Centrifuge Tube
Lo-Bind Microcentrifuge Tube for Cell Count
T75 Flask

All the following steps should take place in a sterile tissue culture hood.

Methods

- 1. Transfer cells from flask or plate into 15 mL centrifuge tube. TIP: Be careful not to create bubbles.
- 2. Add complete RPMI to flask or plate and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
    - For T25 Flask add 2 mL
    - For 6 Well Plate add 1 mL
3. Transfer cell/complete RPMI mixture to the 15 mL centrifuge tube.
- 4. Mix well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
- 5. Take a 10 µL aliquot of your cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
- 6. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
7. Remove cells from centrifuge, check for cell pellet.
- 8. Aspirate supernatant. TIP: Be careful not to aspirate the cell pellet.
    a. Use pipette to remove last bit of supernatant.
- 9. Resuspend target line cells in 1 mL of complete RPMI. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
10. Slowly add complete RPMI to a final concentration of 1 x 10^6 cells/mL.
- 11. Mix with serological pipette by gently pipetting up and down 5 times. TIP: Be careful not to create bubbles.

- 12. Transfer cell suspension to flask. TIP: Slowly pipette down the side of the flask as to not create bubbles.
- 13. Spread out cell suspension by rocking flask carefully to fully cover the bottom of the flask. TIP: Be careful to not make bubbles.
14. Incubate at 37°C, 5% CO_2 and passage the target and control cells as needed over the next few days.
- CRITICAL: Passage sufficient numbers of target and control cells to perform assay at a ratio of 1:2 CAR-T cells to targets.

Chapter 3b: Culture of Adherent Target Cells

Materials Required

Complete RPMI (37°C)
Incubated Cells from Chapter 2
TrypLE Express (Room Temperature)
15 mL Centrifuge Tube
Lo-Bind Microcentrifuge Tube for Cell Count
T75 Flask

All the following steps should take place in a sterile tissue culture hood.

Methods

1. Remove flask or plate containing target cells from incubator.
- 2. Gently aspirate supernatant and any cells in suspension. If target cells are strongly adherent, the supernatant and cells in suspension can be discarded. If target cells are not strongly adherent, it is suggested that the cells in suspension are also collected. CRITICAL: Be careful to not dislodge any cells adhered to the flask or plate.
3. Gently rinse adherent cells once with PBS.
    - For T75 Flask add 3 mL
    - For T25 Flask add 2 mL
    - For 6 Well Plate add 1 mL
4. Aspirate PBS from flask or plate and discard.
5. Detach adherent cells from the flask by adding TrypLE Express.
    - For T75 Flask add 5 mL
    - For T25 Flask add 3 mL
    - For 6 Well Plate add 2 mL
6. Rock the flask back and forth to ensure TrypLE Express covers the entire bottom of the flask.
7. Incubate the cells with TrypLE Express for 5 minutes at 37°C to allow the cells to detach.
8. After incubation, check under microscope to determine if cells are detached.

- If so, proceed to step 9.
- If not, gently tap the flask to dislodge cells and observe under microscope. If cells are still attached, continue to incubate at 37°C and check every 1 to 2 minutes.

9. Gently rinse flask or plate with complete RPMI to collect cells.
    - For T75 Flask add 5 mL
    - For T25 Flask add 3 mL
    - For 6 Well Plate 2 mL
10. Transfer cell/TrypLE/complete RPMI mixture to the 15 mL centrifuge tube.
11. Mix well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
12. Take a 10 µL aliquot of your cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
13. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
14. Remove cells from centrifuge, check for cell pellet.
15. Aspirate supernatant. TIP: Be careful not to aspirate the cell pellet.
    a. Use pipette to remove last bit of supernatant.
16. Resuspend target line cells in 1 mL of complete RPMI. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
17. Slowly add complete RPMI to a final concentration of 1 x 10^6 cells/mL.
18. Mix with serological pipette by gently pipetting up and down 5 times. TIP: Be careful not to create bubbles.
19. Transfer cell suspension to flask. TIP: Slowly pipette down the side of the flask as to not create bubbles.
20. Spread out cell suspension by rocking flask carefully to fully cover the bottom of the flask. TIP: Be careful to not make bubbles.
21. Incubate at 37°C, 5% CO_2 and passage the target and control cells as needed over the next few days.

CRITICAL: Passage sufficient numbers of target and control cells to perform assay at a ratio of 1:2 CAR-T cells to targets.

Chapter 4: Recovery of Cryopreserved CAR-T Cells

Materials Required

Complete RPMI (37°C)
Recombinant IL-2 at 1 µg/mL (-20°C)
15 mL Centrifuge Tube (*Thawed CAR-T*)
Cryopreserved CAR-T Cells
Plate and/or Flask
For > 10 M Cells, T75 Flask
For 6-9.9 M Cells, T25 Flask
For < 6 M, 6 Well Plate

All the following steps should take place in a sterile tissue culture hood.

Methods

1. Pipette 5 mL of complete RPMI into a 15 mL centrifuge tube, labeled *Thawed CAR-T*.
- 2. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. TIP: Be careful of contamination.
3. Quickly move vials into a water bath (37°C) to thaw. While thawing, swirl the vial in the water until a single ice crystal remains in the vial. Be sure to prevent (to the best of your ability) any of the water from the water bath from getting under the cap and into the sample.
4. When the sample is nearly thawed, remove the vial and immediately spray vial with 70% alcohol before bringing into the hood. It is important to allow the alcohol to evaporate before opening the vial.
- 5. Slowly pipette thawed cells into 5 mL of complete RPMI in 15 mL centrifuge tube, labeled *Thawed CAR-T*. TIP: Insert tip into complete RPMI when pipetting, be careful not to create bubbles.
- 6. Take 1 mL of complete RPMI and pipette into original thawed cell vial. Rinse inside the vial with the complete RPMI to recover additional thawed cells. TIP: Insert tip into complete RPMI, be careful not to create bubbles.
- 7. Draw up cell/complete RPMI mixture and pipette into the 15 mL centrifuge tube, labeled *Thawed CAR-T*. TIP: Insert tip into complete RPMI and pipette gently up and down. Be careful not to create bubbles.
8. Centrifuge cells for 10 minutes at 300 rcf.
9. While the cells are centrifuging, take the IL-2 (1 µg/mL) out from -20°C and thaw at room temperature.
- CRITICAL: Use IL-2 aliquot that has been frozen at -20°C for less than a month. Do not use IL-2 that has been previously thawed.
10. Remove cells from centrifuge, check for cell pellet.
- 11. Aspirate supernatant. TIP: Be careful not to aspirate the cell pellet.
    a. Use pipette to remove last bit of supernatant.
- 12. Resuspend cell pellet in 1 mL of fresh complete RPMI. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
13. Slowly add complete RPMI to a final concentration of 1 x 10^6 cells/mL.
14. Mix thawed IL-2 thoroughly by carefully pipetting up and down.

15. Dilute 100 µL of 1 µg/mL IL-2 per 10 mL of cell suspension to a final concentration of 10 ng/mL.

- CRITICAL: Discard thawed IL-2 aliquot if there is any volume remaining. IL-2 must only be thawed once.

16. Mix with serological pipette by gently pipetting up and down 5 times. TIP: Be careful to not create bubbles.
17. Transfer cell suspension to flask or plate. TIP: Slowly pipette down the side of the flask as to not create bubbles.
18. Spread out cell suspension by rocking flask or plate carefully to fully cover the bottom of the container. TIP: Be careful to not make bubbles.
19. Move to incubator for overnight recovery at 37°C, 5% CO_2. NOTE: The time period for overnight recovery is considered 16 – 20 hours, but not exceeding 24 hours.

Chapter 5: Post-Recovery Sample Setup

Materials Required

Complete RPMI (37°C)
15 mL Centrifuge Tube
Overnight Recovered CAR-T Cells from Chapter 4
Lo-Bind Microcentrifuge Tube

All the following steps should take place in a sterile tissue culture hood.

Methods

1. Transfer CAR-T cells from flask or plate into 15 mL centrifuge tube. TIP: Be careful not to create bubbles.
2. Add complete RPMI to flask or plate and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
    - For T25 Flask add 2 mL
    - For 6 Well Plate add 1 mL
3. Transfer cell/complete RPMI mixture to the 15 mL centrifuge tube.
4. Mix with 10 mL serological pipette by gently pipetting up and down 5 times. TIP: Be careful not to create bubbles.
5. Take a 10 µL aliquot of your cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
6. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.

- CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Protocol using Ficoll.

7. Proceed immediately to next chapter.

Chapter 6: CD8 Sample Enrichment

Materials Required

Complete RPMI (37°C)
RoboSep Buffer (4°C)
Miltenyi CD8 Microbeads, Human, 2 mL (4°C)
MACS LS Column
Prepared Cells from Chapter 5
Enrichment Kit:
- MACS Metal Plate/Magnet Kit
- 3 x 15 mL Centrifuge Tubes (*Discard, Flow Through (CD4), CD8 Fraction*)
- Lo-Bind Microcentrifuge Tubes for Post-Enrichment

All the following steps should take place in a sterile tissue culture hood.

Methods

NOTE: If only CD4 cells are required, substitute the Miltenyi CD8 Microbeads in this chapter for Miltenyi CD4 Microbeads.

1. Remove cells from centrifuge and check for cell pellet.
2. Aspirate supernatant. TIP: Be careful not to aspirate the cell pellet.
    a. Use pipette to aspirate remaining supernatant.

CRITICAL: For every 1 x 10^7 cells, resuspend in 80 µL RoboSep (4°C) and 20 µL of CD8 beads (4°C).

3. Add 80 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
4. Vortex Miltenyi CD8 Microbeads at a slow speed for 10 seconds.
5. Add 20 µL of Miltenyi CD8 Microbeads and mix well by gently pipetting up and down 5 times.

TIP: Make sure to eliminate clumps so that beads are evenly distributed among cells. Be careful not to create bubbles.

6. Incubate in refrigerator (4°C) for 15 minutes. TIP: Don't incubate on ice as increased incubation times may be required.
7. After 15 minutes, add 2 mL of cold RoboSep. TIP: Not necessary to mix for this step.
8. Centrifuge cells for 10 minutes at 300 rcf.

TIP: Keep RoboSep in refrigerator during enrichment process.

9. Set up MACS sorting by setting metal plate in tissue culture hood and placing magnet on metal plate. Place LS column in magnet with wings facing out and align the 15 mL centrifuge tube labeled "Discard" under the LS column. CRITICAL: LS Column should not touch the tubes.
10. After cells are centrifuged, check for cell pellet and continue with MACS separation.

- 11. Aspirate RoboSep from cell pellet. TIP: Since it is a small volume, use pipette for this step to prevent accidental aspiration of the cell pellet.
12. For 1 x 10^8 or fewer cells, resuspend with 500 µL of cold RoboSep.
    - a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
- CRITICAL: Be careful not to let column dry out. Make sure not to add liquid when there is already liquid in the LS Column.
- 13. Starting with the LS column over the "Discard" tube, add 3 mL of cold RoboSep to LS Column. CRITICAL: Be careful not to create bubbles or touch sides of LS column. Let all the RoboSep flow through before moving on to next step. As a reminder, be careful to not let the column dry out.
14. Unscrew and keep cap for "Flow Through (CD4)" tube. NOTE: This is in preparation for next step to ensure the column does not dry out during the transition.
- 15. When last drop falls through to "Discard" tube, move the rack over so the LS column is over the flow through tube. CRITICAL: Be careful not to let column dry. If there is one drop remaining that will not fall, move on to next step.
16. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
17. Mix cell suspension by gently pipetting up and down 5 times. NOTE: This ensures that the cells are evenly dispersed after sitting.
18. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching sides.
19. Wash 3 times with 3 mL of cold RoboSep.
    - a. First wash: Rinse inside walls of cell suspension tube with 3 mL of RoboSep before transferring the mixture to LS Column. NOTE: This is to retrieve any cells that have been left behind.
    - i. Pipette all the mixture into LS Column after last drop passes through or does not fall from step 18. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.
    - b. Second wash: Add 3 mL of RoboSep into LS Column after last drop passes through or does not fall. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.
    - c. Third wash: Add 3 mL of RoboSep into LS Column after last drop passes through or does not fall. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.
20. After last drop of the third wash passes through or does not fall, remove the LS Column carefully from the magnet, and place carefully on the tube labeled for "CD8 fraction".
21. Cap the "Flow Through (CD4)" tube. This will be used for CD4 CAR-T cell subset in Cell Stimulation (Chapter 7). Do not discard.
- 22. Add 5 mL of cold RoboSep to the LS column. CRITICAL: Be careful not to touch the sides.
- 23. Take plunger, smoothly push down on the plunger to push the RoboSep buffer through the LS Column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.
24. Set LS Column back on the "CD8 fraction" tube.

25. Loosen up plunger. Remove plunger briefly from column and hold in one hand.

- CRITICAL: Do not allow the plunger to interact with external contaminants. It will be used for one more step.

26. Add another 4 mL of cold RoboSep to the LS Column.

- 27. Take plunger, smoothly push down on the plunger to push the RoboSep buffer through the LS Column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.

28. Discard LS Column and plunger.

29. Centrifuge "CD8 Fraction" tube and "Flow Through (CD4)" tube for 10 minutes at 300 rcf.

30. Remove cells from centrifuge, check for cell pellets.

- 31. Aspirate RoboSep buffer from "CD8 fraction" and "Flow Through (CD4)" tubes. TIP: Be careful to not aspirate cell pellet.
- 32. Use pipette to aspirate the remaining supernatant from each tube. TIP: Be careful to not aspirate cell pellet.

33. Gently resuspend in 500 µL of complete RPMI.

- a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure there are no clumps or bubbles.

- 34. Take a 10 µL aliquot of cell fractions and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
- 35. Centrifuge cells for 10 minutes at 300 rcf. While cells are spinning down, use hemocytometer to get cell counts. CRITICAL: See Appendix D1 for cell counting instructions.

36. Remove cells from centrifuge, check for cell pellets.

- 37. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cells.

38. Gently resuspend cell fractions at a density of 1 x 10^6 cells/mL in complete RPMI.

- a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.

39. Incubate cell suspensions at 37°C 5% CO_2 until Cell Stimulation (Chapter 7).

Chapter 7: Cell Stimulation

NOTE: Please read before proceeding with next chapter.

There are 2 sections depending on your type of target cell:

1. Stimulation with suspension target cells as described in Chapter 7a.
2. Stimulation with adherent target cells as described in Chapter 7b.

Chapter 7a: Cell Stimulation with Suspension Target Cells

Materials Required

Complete RPMI (37°C)
2 x 15 mL Centrifuge Tubes
96 Well Plate U-Bottom
CAR-T Specific Target Cell Culture(s) from Chapter 2
CD8 Fraction
Flow Through (CD4)
2 x Lo-Bind Microcentrifuge Tubes

All the following steps should take place in a sterile tissue culture hood.

Methods

- 1. Mix target cell line(s) gently up and down using a pipette. TIP: Be careful not to create bubbles.
- 2. Transfer target cells from flask into 15 mL Centrifuge Tube. TIP: Be careful not to create bubbles.
- 3. Add complete RPMI to flask and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
- 4. Transfer target cells/complete RPMI mixture to the 15 mL Centrifuge Tube. TIP: Be careful not to create bubbles.
- 5. Take a 10 µL aliquot of target cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
- 6. Centrifuge cells for 10 minutes at 300 rcf. While cells are spinning down, use hemocytometer to get cell counts. CRITICAL: See Appendix D1 for cell counting instructions.

CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Protocol using Ficoll.

7. Remove cells from centrifuge, check for cell pellet.
- 8. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
- 9. Resuspend target cells with complete RPMI to a cell concentration of 2 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.
10. Using a P100 pipette, add 100 µL of CAR-T specific target cells and control cells, if applicable, to desired number of wells in the 96 Well U-Bottom Plate. NOTE: Concentration is 2 x 10^6 cells/mL.

CRITICAL: Use a 1:1 volume ratio for the overall density ratio of 1 CAR-T cell to 2 CAR-T specific target cells. Volume in each well should be 200 µL.

11. Add 100 µL of the CD8 CAR-T cells to the well(s) containing the CAR-T specific target cells and control cells, if applicable, in the 96 Well U-Bottom Plate. NOTE: Concentration is 1 x 10^6 cells/mL.

- a. Mix cell suspension thoroughly. TIP: Be careful not to create bubbles. Pipette thoroughly, but gently.

12. Repeat step 11 with the CD4 CAR-T cells.

- CRITICAL: Be careful not to create bubbles. This will maximize even stimulation of cell suspension.

13. Incubate 96 Well Plate U-Bottom for 20 hours at 37°C, 5% CO_2.

Chapter 7b: Cell Stimulation with Adherent Target Cells

Materials Required

Complete RPMI (37°C)
Sterile 1X PBS (Room Temperature)
2 x 15 mL Centrifuge Tubes
CAR-T Specific Target Cell Culture(s) from Chapter 2
Appropriate Culture Plate
CD8 Fraction
Flow Through (CD4)
TrypLE Express (Room Temperature)
2 x Lo-Bind Microcentrifuge Tubes

All the following steps should take place in a sterile tissue culture hood.

Methods

1. Remove flask containing target cell line(s) from incubator.
2. Aspirate supernatant and any cells in suspension. If target cells are strongly adherent, the supernatant and cells in suspension can be discarded. If target cells are not strongly adherent, it is suggested that the cells in suspension are also collected. CRITICAL: Be careful to not dislodge any cells adhered to the flask or plate.
3. Gently rinse adherent cells once with PBS.
    - For T75 Flask add 3 mL
4. Aspirate PBS from flask and discard.
5. Detach adherent cells from the flask by adding TrypLE Express.
    - For T75 Flask add 5 mL
6. Rock the flask back and forth to ensure TrypLE Express covers the entire bottom of the flask.
7. Incubate the cells with TrypLE Express for 5 minutes at 37°C to allow the cells to detach.
8. After incubation, check under microscope to determine if cells are detached.
    - If so, proceed to step 9.
    - If not, gently tap the flask to dislodge cells and observe under microscope. If cells are still attached, continue to incubate at 37°C and check every 1 to 2 minutes.
9. Gently rinse flask with complete RPMI to collect cells.
    - For T75 Flask add 5 mL

- 10. Transfer target cells/TrypLE/complete RPMI mixture to the 15 mL Centrifuge Tube. TIP: Be careful not to create bubbles.
- 11. Take a 10 µL aliquot of target cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
- 12. Centrifuge cells for 10 minutes at 300 rcf. While cells are spinning down, use hemocytometer to get cell counts. CRITICAL: See Appendix D1 for cell counting instructions.
- CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Protocol using Ficoll.

13. Remove cells from centrifuge and check for cell pellet.

- 14. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.

15. Resuspend target cells with complete RPMI to a cell concentration of 2 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.

16. Using a pipette, add target cells into a well of an appropriately sized plate as per Table 11.

Table 11: Volume Required for Different Plate Formats

Plate	Volume
48 Well Plate	500 µL/well
24 Well Plate	1 mL/well
6 Well Plate	2 mL/well

17. Incubate cells for 2 hours, or longer if required, at 37°C, 5% CO_2, until cells are beginning to adhere to plate.

- CRITICAL: Use a 1:1 volume ratio for an overall density ratio of 1 CAR-T cell to 2 target cells.

18. Add CD8+ CAR-T cells to the plate prepared with target cells at a 1:1 volume ratio for an overall density ratio of 1 CAR-T cell to 2 target cells.

19. Repeat step 18 with CD4+ CAR-T cells.

- OPTIONAL: Centrifuge the plate for 5 minutes at 300 rcf to bring CAR-T cells to bottom of wells.

20. Incubate the co-culture for 20 hours at 37°C, 5% CO_2.

Chapter 8: Target Cell Removal

NOTE: Please read before proceeding with next chapter.

There are 2 sections depending on your type of target cell:

1. Removal of suspension target cells as described in Chapter 8a.
2. Removal of CAR-T cells from adherent target cells as described in Chapter 8b.

Chapter 8a: Suspension Target Cell Removal

Materials Required

96 Well Plate Containing CD8 CAR-T Co-Cultures and CD4 CAR-T Co-Cultures
Sterile 1X PBS (Room Temperature)
EasySep Magnets
2 x 15 mL Centrifuge Tubes
4 x Lo-Bind Microcentrifuge Tubes
2 x 5 mL Polystyrene Tubes
Target Cell Line Depletion Beads (4°C)

All the following steps should take place in a sterile tissue culture hood.

Methods

- CRITICAL: Target cell line depletion beads will need to be prepared prior to starting Chapter 8a. See Appendix D3 for instructions. The protocol described in Appendix D3 describes how to conjugate depletion beads for two widely used target cells; K562 cells (CD235a) and Raji cells (CD19). These depletion beads can be used for any target cells expressing CD235a or CD19 respectively.

1. Resuspend CD8 CAR-T cells by pipetting up and down before transferring into a Lo-Bind Microcentrifuge Tube. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
2. Repeat step 1 using CD4 CAR-T cells.
3. Centrifuge cells for 10 minutes at 300 rcf.
4. Remove cells from centrifuge, check for cell pellet.
5. Aspirate supernatant with a pipette.* TIP: Be careful not to aspirate the cell pellets.

   *NOTE: Supernatants may be stored at -80°C for bulk assay.

6. Remove depletion beads from 4°C and resuspend beads using a P1000 pipette. TIP: Make sure to eliminate clumps so that beads are evenly distributed among cells. Be careful not to create bubbles.

- CRITICAL: For every 3 x 10^5 cells, add 50 µL of depletion beads (e.g., antiCD235a-conjugated beads for K562 cell depletion) to each cell pellet and return remainder of beads to 4°C.

   a. Mix well to resuspend. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.

7. Incubate for 10 minutes at room temperature.
8. Gently mix with a P100 pipette to ensure beads are kept in suspension. CRITICAL: Resuspend every 2 minutes to keep mixture in suspension.

9. After the incubation, add 950 µL of PBS to the CD8 CAR-T cells. Repeat step using CD4 CAR-T cells.
10. Gently pipette up and down to resuspend. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
11. Transfer CD8 CAR-T mixture from Lo-Bind Microcentrifuge Tube to a pre-labeled 5 mL polystyrene tube. Repeat step using CD4 CAR-T cells.
12. Place 5 mL polystyrene tubes in EasySep magnets for 2 minutes.
13. After 2 minutes, keep 5 mL polystyrene tubes attached to the magnets and forcefully decant cells into 15 mL centrifuge tubes. CRITICAL: Be careful not to disrupt the 5 mL polystyrene tube from magnet.
14. Remove 5 mL polystyrene tubes from the EasySep magnets.
15. Add 1 mL of PBS to the empty Lo-Bind Microcentrifuge Tubes from step 11 to recover any remaining material.
16. Transfer contents to corresponding 5 mL polystyrene tubes. NOTE: Make sure to rinse the walls of 5 mL polystyrene tubes well.
17. Place 5 mL polystyrene tubes in an EasySep magnets for 2 minutes.
18. After 2 minutes, keep 5 mL polystyrene tubes attached to the magnets and forcefully decant cells into 15 mL centrifuge tubes. CRITICAL: Be careful not to disrupt the 5 mL polystyrene tube from magnet.

    NOTE: Pool target cell depleted T cell suspensions from the same sample into one 15 mL centrifuge tube.
19. Centrifuge CAR-T cells for 10 minutes at 300 rcf.
20. Remove cells from centrifuge, check for cell pellets.
21. Aspirate supernatant. TIP: Be careful not to aspirate cell pellets.
    a. Use pipette to aspirate remainder of supernatant.
22. Gently resuspend in 500 µL of complete RPMI.
    a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
23. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
24. Draw up all 500 µL of target depleted CD8 CAR-T cells and pipette into a Lo-Bind Microcentrifuge Tube. Repeat step with CD4 CAR-T cells.
25. Centrifuge CAR-T cells for 10 minutes at 300 rcf.

Chapter 8b: Adherent Target Cell Removal

Materials Required

Flask or Plate Containing CD8 CAR-T Co-Cultures and CD4 CAR-T Co-Cultures
4 x 15 mL Centrifuge Tubes
4 x Lo-Bind Microcentrifuge Tubes
Complete RPMI (37°C)

All the following steps should take place in a sterile tissue culture hood.

Methods

- 1. Carefully pipette CD8 CAR-T cells in suspension and transfer to a 15 mL centrifuge tube. CRITICAL: Be careful to not dislodge any cells adhered to the plate. NOTE: Pool T cell suspensions from the same sample into one 15 mL centrifuge tube.
2. Repeat step 1 using CD4 CAR-T cells.
3. Centrifuge cells for 10 minutes at 300 rcf.
4. Remove cells from centrifuge, check for cell pellet.
- 5. *Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.

   *NOTE: Supernatants may be stored at -80°C for bulk assay.
6. Gently resuspend in 500 µL of complete RPMI.
- a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
7. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
8. Draw up all 500 µL of target depleted CD8 CAR-T cells and pipette into a Lo-Bind Microcentrifuge Tube. Repeat step with CD4 CAR-T cells.
9. Centrifuge CAR-T cells for 10 minutes at 300 rcf.

Chapter 9: Chip Thawing

Materials Required

IsoCode Chips in Vacuum Sealed Bag (-20°C)

Methods

- 1. Take vacuum sealed bag containing IsoCode chips from -20°C. CRITICAL: Chips must stay sealed until Chip Loading (Chapter 11).
2. Place on a bench to thaw at ambient temperature 30-60 minutes prior to use.
3. While chips thaw, prepare liquid reagents and setup in the Bruker instrument. Refer to your instrument's system guide for detailed instructions.

Chapter 10: Cell Staining

Materials Required

Complete RPMI (37°C)
AF647 anti-human CD8 (4°C)
AF647 anti-human CD4 (4°C)
CD8 CAR-T Cells
CD4 CAR-T Cells
4 x Lo-Bind Microcentrifuge Tubes (*CD8 CAR-T Control Cells, CD8 CAR-T Target Cells, CD4 CAR-T Control Cells, CD4 CAR-T Target Cells*)

All the following steps should take place in a sterile tissue culture hood.

Methods

1. Remove cells from centrifuge, check for cell pellets.
2. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
3. CRITICAL: For every 1 x 10^5 cells, resuspend in 18 µL of complete RPMI and 2 µL of appropriate surface marker stain.
4. Gently resuspend in 18 µL of complete RPMI for 1 x 10^5 or fewer cells.
    a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
5. Centrifuge tubes of AF647 anti-human CD8 stain & AF647 anti-human CD4 stain in a micro centrifuge for 10 seconds to collect the stain at the bottom of the tubes.
6. Add 2 µL (1:10 final dilution) of AF647 anti-human CD8 to CD8 CAR-T cells for 1 x 10^5 or fewer cells. Mix gently by pipetting up and down. CRITICAL: Be sure to use appropriate stain for each cell subset.
7. Add 2 µL (1:10 final dilution) of AF647 anti-human CD4 to CD4 CAR-T cells for 1 x 10^5 or fewer cells. Mix gently by pipetting up and down. CRITICAL: Be sure to use appropriate stain for each cell subset.
8. Incubate stained CD8 CAR-T cells and CD4 CAR-T cells for 20 minutes at room temperature in the dark.
9. After 20 minutes, add 1 mL of complete RPMI to the stained cells. TIP: Mix gently, be careful not to create bubbles.
10. Centrifuge stained cells for 10 minutes at 300 rcf.
11. Remove cells from centrifuge, check for cell pellets.
12. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
13. Gently resuspend in 500 µL of complete RPMI.
    a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
14. Take a 10 µL aliquot of stained CD8 CAR-T cells and stained CD4 CAR-T cells, transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

14. Centrifuge stained cells for 10 minutes at 300 rcf. While cells are spinning down, use hemocytometer to get cell counts. CRITICAL: See Appendix D1 for cell counting instructions.
15. Remove cells from centrifuge, check for cell pellets.
16. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
17. Gently resuspend CAR-T cells at 1 x 10^6 cells/mL in complete RPMI.
    a. Mix well to resuspend. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
18. Incubate stained CD8 CAR-T cells and CD4 CAR-T cells at 37°C 5% CO_2 until Chip Loading (Chapter 11).

Chapter 11: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 9
CD8 CAR-T Cells at 1 x 10^6 Cells/mL
CD4 CAR-T Cells at 1 x 10^6 Cells/mL

Methods

NOTE: CD4 CAR-T cells and CD8 CAR-T cells should be loaded on separate chips as the stain will not distinguish between the two types.

1. Remove IsoCode chips from vacuum sealed bag and place on a flat surface. CRITICAL: Keep protective blue film on bottom.
2. Resuspend CD8 CAR-T cells by pipetting up and down. Pipette 30 µL of cell suspension into IsoCode chip. CRITICAL: Be careful not to create bubbles. Insert pipette tip vertically into inlet port until tip lightly touches bottom, and slowly pipette 30 µL into inlet port. Be careful not to eject second step of pipette–it will cause bubbles.
3. Resuspend CD4 CAR-T cells by pipetting up and down. Pipette 30 µL of cell suspension into IsoCode chip. CRITICAL: Be careful not to create bubbles. Insert pipette tip vertically into inlet port until tip lightly touches bottom, and slowly pipette 30 µL into inlet port. Be careful not to eject second step of pipette–it will cause bubbles.
4. Let IsoCode chips sit for one minute on a flat surface.
5. Check bottom of chip to ensure liquid has entered the chip. TIP: If liquid has not flowed, tap IsoCode chip on flat surface lightly.
6. When inserting IsoCode chip into instrument, make sure the logo is facing up and towards you with the magnet facing the instrument. Take the blue film off while inserting each IsoCode chip into the instrument.

   NOTE: Please refer to your instrument's loading instructions for details.

D. Appendix

D1 Protocol: Cell Quantification & Viability

Hemocytometer
10 µL aliquot of cells
Trypan Blue

NOTE: Automated cell counters can be used in this protocol EXCEPT prior to loading cells on chip due to spectral overlap of the stains. Manual cell counting is required prior to loading on the chip.

NOTE: To obtain an accurate representation of cell viability, cells should be counted within 15 minutes of staining as cell viability will drop over time because Trypan Blue is toxic.

1. Quick spin the Trypan Blue to pellet potential debris. Remove aliquot from the top of Trypan Blue.
2. Using a P10 pipette, add equal volume of Trypan blue solution to 10 µL of sample. Mix gently to resuspend.
   TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
3. Load onto hemocytometer. CRITICAL: Be careful not to overfill or create bubbles.
4. Count and record viable (clear) and dead cells (blue) of all four 16-square corners.
   CRITICAL: If more than 200 cells/16 squares were counted, repeat count using a 1:5 or 1:10 dilution with 1X PBS or complete RPMI using a fresh sample aliquot.
5. Calculate the concentration of cells as follows:
   a. Concentration (cells/mL) = Average per square cell count x 10^4 x dilution factor
6. Calculate the number of cells as follows:
   a. Number of cells = Cell concentration (cells/mL) from D.1.5 x total volume of cell suspension (mL)
7. Calculate percent viable cells:
   a. % Viable cells = 100 x number of viable cells / [number of viable cells + number of dead cells]

D2 Protocol: Dead Cell Removal Using Ficoll

Materials Required

Complete RPMI (37°C)
Cells (Minimum 3 x 10^6)
2 x 15 mL Centrifuge Tubes
Lo-Bind Microcentrifuge Tube(s)
Ficoll Paque (Room Temperature)

- CRITICAL: It is recommended to start this protocol with a minimum of 3 x 10^6 total cells.

1. Carefully add 6 mL of Ficoll to the bottom of the required amount of 15 mL centrifuge tube(s) prior to harvesting cultures.
2. Centrifuge cells for 10 minutes at 300 rcf.
3. Remove cells from centrifuge, check for cell pellet.

- 4. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.

a. Use pipette to aspirate remaining supernatant.

- 5. Resuspend the pellet(s) in 7 mL of complete RPMI. TIP: Be careful not to create bubbles.
- a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
- CRITICAL: Do not use more than 1 x 10^7 cells of your suspension per Ficoll tube.
- 6. Add the cell suspension(s) VERY SLOWLY to the tube(s) containing Ficoll. CRITICAL: Place the tip of your pipette on the wall of the tube, close to the Ficoll layer. Add cell suspension VERY SLOWLY.
- CRITICAL: This step must be done carefully and slowly to avoid mixing of the layers.
- 7. Centrifuge tubes for 20 minutes at 300 rcf without break or acceleration. CRITICAL: Turn acceleration and brakes off to preserve the density layers established during centrifugation.

8. While cells centrifuge, prepare appropriate amount of 15 mL centrifuge tube(s) containing 6 mL of complete RPMI.
9. Remove cells from centrifuge, check for cloudy layer which are the viable cells.

- 10. Aspirate a small volume of the supernatant. CRITICAL: Be careful not to aspirate cloudy layer containing cells.

11. Using a P1000 pipette, collect the viable cells by recovering the cloudy layer between Ficoll and complete RPMI media.
12. Transfer cells into the 15 mL centrifuge tube(s) containing complete RPMI.

- 13. Aliquot 10 µL of cell/complete RPMI mixture(s) into a Lo-Bind Microcentrifuge Tube(s) and proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.

D3 Protocol: Bulk Depletion Bead Preparation

Materials Required

Dynabeads M-280 Streptavidin (4°C)
Biotin Anti-Human CD235a or Biotin Anti-Human CD19 Antibody (4°C)
Sterile 1X PBS (Room Temperature)
0.1% BSA (Room Temperature)
EasySep Magnet
1 x 15 mL Centrifuge Tube

1 x 5 mL Polystyrene Tube

NOTE: This protocol produces 2 mL of conjugated depletion beads.

Table 12: Antibodies Used for Specific Target Cell Lines

Target Cell Line	Antibody Needed
K562	Biotin Anti-Human CD235a
Raji	Biotin Anti-Human CD19

1. Remove Dynabeads M-280 from 4°C.
2. Using a P1000, resuspend Dynabeads M-280 well. NOTE: Dynabeads M-280 will sit pelleted at the bottom of the container. Resuspension should achieve a solid-colored mixture.
3. Pipette 500 µL of beads into a 5 mL polystyrene tube. Return any remaining Dynabeads to 4°C for storage according to manufacturer's protocol.
4. Pipette 1 mL of PBS into the 5 mL polystyrene tube and gently mix up and down 3-4 times.

   NOTE: Mixing removes any remaining sodium azide from the manufacturer's storage buffer.
5. Place the 5 mL polystyrene tube in an EasySep Magnet for 1 minute.
6. Gently decant supernatant into a 15 mL centrifuge tube. CRITICAL: Do not disturb the polystyrene tube in magnet. Doing so will transfer beads into decanted supernatant.
7. Remove the 5 mL polystyrene tube from EasySep Magnet.
8. Repeat steps 4-7 one more time for a total of two washes.
9. Pipette 100 µL of antibody into the 5 mL polystyrene tube. Return any remaining antibody to 4°C for storage according to manufacturer's protocol. NOTE: Type of antibody (CD235a or CD19) will be dependent on target cell line. Refer to Table 12.
10. Mix well to ensure antibody and beads are in suspension by gently pipetting up and down 3-4 times.
11. Incubate 5 mL polystyrene tube for 30 minutes at room temperature in the dark.
    a. With a P100, pipette mixture every 5 minutes to keep beads in suspension.
12. After 30 minutes, add 1 mL of 0.1% BSA to 5 mL polystyrene tube and mix by gently pipetting up and down 3-4 times.
13. Place the 5 mL polystyrene tube in an EasySep Magnet for 1 minute.
14. Gently decant supernatant into a 15 mL centrifuge tube. CRITICAL: Do not disturb the polystyrene tube in magnet. Doing so will transfer beads into decanted supernatant.
15. Remove the 5 mL polystyrene tube from EasySep Magnet.
16. Add 1 mL of 0.1% BSA to the 5 mL polystyrene tube, making sure to rinse the beads from the side of the tube, and mix by gently pipetting up and down 3-4 times.
17. Place the 5 mL polystyrene tube in an EasySep Magnet for 1 minute.

18. Gently decant supernatant into a 15 mL centrifuge tube. CRITICAL: Do not disturb the polystyrene tube in magnet. Doing so will transfer beads into decanted supernatant.
19. Repeat steps 15-18 three more times for a total of five washes.
20. Resuspend conjugated beads in 2 mL of 0.1% BSA.
21. Seal the 5 mL polystyrene tube by capping the tube and tightly wrapping the capped tube with parafilm.
22. Store conjugated bead mixture upright in the 5 mL polystyrene tube at 4°C.

    NOTE: Conjugated depletion beads can be stored at 4°C for up to three months.

D4 Protocol: Test Depletion Beads (Optional)

Materials Required

Anti-Human CD235a or Anti-Human CD19 Conjugated Depletion Beads (4°C)
T Cells or CAR-T Cells
Target Cells
96 Well Plate U-Bottom
15 mL Centrifuge Tube
Lo-Bind Microcentrifuge Tubes

NOTE: It is recommended to complete this section to demonstrate the efficiency of depletion beads produced according to the protocol in Appendix D3 before proceeding to your experiment.

NOTE: CAR-T or T Cells used in this Appendix could be the same cells that are intended for use with this protocol or healthy control cells. It is recommended to test the same cell subset that will be run in the protocol. For instance, if you intend to assay CD8+ CAR-T cells on the Bruker instrument then it is recommended to use CD8+ CAR-T cells in this depletion bead test.

Before starting this test, obtain an enriched T cell or CAR-T cell population. Refer to Chapter 6 or PRO-1 Chapters 5 and 6 for instructions. Ensure enriched CAR-T or T cell population is suspended at 1 x 10^6 cells/mL prior to starting this protocol. Additionally, ensure target cells are available for this test. Refer to Chapters 2 and 3 for instructions.

1. Remove target cell line(s) from the incubator.
2. Mix target cell line(s) gently up and down using a pipette. TIP: Be careful not to create bubbles.
3. Transfer target cells from flask into 15 mL Centrifuge Tube. TIP: Be careful not to create bubbles.
4. Add complete RPMI to flask and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
5. Transfer target cells/complete RPMI mixture to the 15 mL Centrifuge Tube. TIP: Be careful not to create bubbles.
6. Take a 10 µL aliquot of target cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.

- 7. Centrifuge cells for 10 minutes at 300 rcf. While cells are spinning down, use hemocytometer to get cell counts. CRITICAL: See Appendix D1 for cell counting instructions.
- CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Protocol using Ficoll.

8. Remove cells from centrifuge and check for cell pellet.

- 9. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
- 10. Resuspend target cells with complete RPMI to a cell concentration of 2 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.

Table 13: Test Conditions for Bulk Depletion Beads

Condition	To be Depleted?	Expectation for Test
Target Cells Alone	Yes	>80% of target cells depleted
Target Cells & CAR-T Cells (or T Cells)	Yes	>80% of target cells depleted Low depletion of CAR-T cells (or T cells), cannot exceed 50%
Target Cells & CAR-T Cells (or T Cells)	No	Both target cells and CAR-T cells (or T cells) remain
CAR-T Cells (or T Cells) Alone	Yes	Low depletion of CAR-T cells (or T cells), cannot exceed 50%

11. Using a P100 pipette, add 100 µL of target cells to desired number of wells on the 96 well U-bottom plate. NOTE: Concentration is 2 x 10^6 cells/mL. At minimum, 1 well should be plated for each target cell containing condition (minimum of 3 wells).
12. Using a P100 pipette, add 100 µL of pre-enriched CAR-T cells (or T cells) to desired number of wells containing target cells in the 96 well U-bottom plate. NOTE: Concentration is 1 x 10^6 cells/mL. Total volume in each well will be 200 µL.

- CRITICAL: Do not add CAR-T cells (or T cells) to at least one well on the 96 well U-bottom plate. The well(s) will serve as the target cells alone condition.

13. Using a P100 pipette, add 100 µL of pre-enriched CAR-T cells (or T cells) to desired number of **empty** wells on the 96 well U-bottom plate. NOTE: Concentration is 1 x 10^6 cells/mL.

- 14. Mix all cell suspensions thoroughly. TIP: Be careful not to create bubbles. Pipette thoroughly, but gently.

15. Incubate 96 Well Plate U-Bottom for 20 hours at 37°C, 5% CO_2. NOTE: 1 hour incubation can be used as an approximation if needed.
16. Deplete cells for the conditions outlined in Table 13. Refer to Chapter 8a steps 1 through 24.

17. After depletion, take a 10 µL aliquot of each of the 4 conditions and transfer to a Lo-Bind microcentrifuge tube for cell counting and visual interpretation. CRITICAL: See Appendix D1 for cell counting instructions. Cell counting must be completed manually using a hemocytometer to allow for visual inspection of each condition. Target cell type dependent, cell size or cell morphology can be used to distinguish between the target cells and the CAR-T cells (or T cells).
18. Refer to Table 13 for expectations.

    For assistance in optimizing your depletion beads or any other steps in the assay, please contact your local Field Applications Scientist or the Scientific Support team. Contact information for the Scientific Support team can be found in the Troubleshooting and References section.

Troubleshooting & References

Please contact Support at 844-476-7539 (toll free) or 475-221-8402 or email support@isoplexis.com with specific troubleshooting questions.

Problem	Possible Reason	Solution
Low quality cell count on chip *Cell Counting & Concentration related*	• Recommended cell concentrations not used • Issue with Cell Counting procedure • Trypan Blue may have debris • Trypan Blue is toxic • Poor cell removal from 96 well U-bottom plate	• Use recommended cell concentrations during overnight incubation (**Chapter 7**) • Use appropriate dilutions recommended in **Appendix D1** • Do a recount if initial count does not seem accurate • Quick spin Trypan Blue to pellet potential debris, remove aliquot from top of Trypan Blue. Start with fresh aliquot of Trypan Blue. • Count within 15 minutes of staining cells • Thoroughly mix cells in well with pipette prior to transferring to tube (refer to **steps 8a.1 and 8a.2**)
Low quality cell count on chip *Stain Process related*	• Use of media other than the recommended media in protocol which could interact with cell stain • Use of stains not recommended in protocol • Recommended stain concentration, incubation time and/or incubation temperature not used	• Use complete RPMI media following recipe in **Table 7** • Use Bruker provided validated stains (**Table 4: Cell Staining Reagents**) • Follow staining steps as highlighted in **Chapter 10**

Low quality cell count on chip *Technique Detail related*	• Bubbles loaded onto chip, especially at Chip Loading
	• Detection of potential artifacts such as debris, cell clumping, inefficient enrichment possibly due to:
	  ○ Pipetting wrong concentration
	  ○ Not fully inserting column into MACS separator
	  ○ Reagents not stored at recommended temperatures
	• Recommended number of cells not loaded on chip
	• Cell pellet or cells lost during centrifuging
		• Follow Critical step in **11.2** to avoid introduction of bubbles on chip
		• Ensure use of a sterile space to reduce introduction of potential contaminants. Use dedicated pipettes, tips, and tubes for sterile work. Pipette up and down gently and throughout protocol to reduce clumps.
		• Follow closely the Critical steps and tips in **Chapter 6** (CD8 Enrichment)
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 11**)
		• Use low protein binding centrifuge tubes

Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Viability related*	• Leaving thawed cells in DMSO for an extended period • Low viable cells due to low viability input sample and lack of utilization of Ficoll-Paque • Decreased viability due to cell shock	• After thaw, quickly transfer cells from DMSO to complete RPMI to ensure viability of cells. • Verify viability of cells is above 80% as stated in **Chapters 5 and 7** to ensure protocol is being performed with the highest quality of cells. Use Ficoll Paque in **Appendix D2** if viability is less than 80% • Use reagents at recommended temperatures (i.e. always use warmed media [37°C])

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended CAR-T to target cell ratio was not used • Recommended incubation duration was not used	• Use recommended CAR-T to target cell ratio as listed in **steps 7a.10 and 7a.11 or 7b.17 and 7b.18** • Use incubation timing listed in **Chapter 7**