Store at 4°C

CAR (Whitlow/218 Linker) Cell Enrichment Kit

#65686

1 Kit
(~2 x 10^9 total input cells)

Cell Signaling TECHNOLOGY®

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For Research Use Only. Not for Use in Diagnostic Procedures.

Product Includes	Product #	Kit Quantity	Storage Temp
Lyophilized DNase I	91019	3 x 1 ea	4°C
Releasable Streptavidin Beads	27119	1 x 5 mL	4°C
DNase I Reconstitution Buffer	47776	1 x 2 mL	4°C
Biotinylated Whitlow/218 Linker (E3U7Q) Rabbit mAb	24801	2 x 1,250 µL	4°C

Description: The CAR (Whitlow/218 Linker) Cell Enrichment Kit is designed to positively select cells that are engineered to express a Whitlow/218 linker-containing single-chain variable fragment (scFv)-based chimeric antigen receptor (CAR) on the cell surface. Using an indirect magnetic bead-based immunoaffinity enrichment protocol, this kit yields highly pure and viable CAR positive cells that are bead-free. Within a heterogeneous population of cells, desired CAR expressing cells are labeled with a biotinylated rabbit monoclonal antibody targeting the Whitlow/218 linker. Releasable Streptavidin Beads are then incubated with the cell mixture. Bead-bound cells are isolated without columns using a magnet (not included), and then beads are removed from cells using DNase I. Following release from beads, the positively selected fraction of CAR positive cells containing surface-bound biotinylated antibody-streptavidin complexes are available for expansion or use in downstream applications such as flow cytometry and gene expression analyses.

Background: Magnetic bead-based immunoaffinity cell enrichment is a gentle purification method that can be leveraged, in part, to facilitate a robust interrogation of the biology of immune cells that are engineered to express CARs. For example, isolation of rare subsets of CAR positive cells from a complex, heterogeneous population of cells presents the opportunity for extensive downstream analyses using single-cell omics assays. In the context of CAR cell engineering, magnetic bead-based immunoaffinity cell enrichment can also be useful in scenarios when delivery of the CAR transgene is inefficient, resulting in a small population of cells expressing the CAR transgene.

Specificity/Sensitivity: Biotinylated Whitlow/218 Linker (E3U7Q) Rabbit mAb recognizes exogenously expressed levels of scFv-based CARs containing a Whitlow/218 linker.

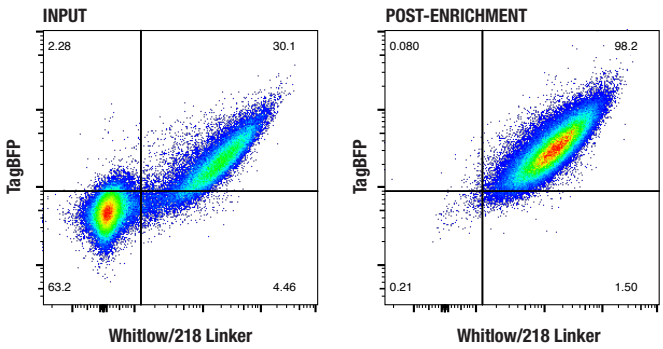
Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to residues of the Whitlow/218 linker peptide and then conjugated to biotin under optimal conditions. Biotinylated antibody endotoxin levels are < 0.1 EU / µg.

Storage: All components in this kit are stable in accordance with the date printed on the outer packaging label when stored at the recommended temperature.

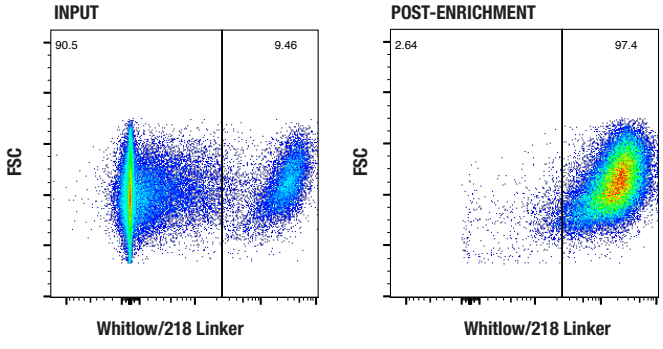
Releasable Streptavidin Beads are supplied as 4 × 10^8 beads/mL in phosphate buffered saline (PBS), pH 7.4, containing 0.1% Tween and 0.02% sodium azide as a preservative.

Biotinylated Whitlow/218 Linker (E3U7Q) Rabbit mAb is supplied in PBS (pH 7.2), less than 0.1% sodium azide, and 2 mg/mL BSA. Store at 4°C. *Do not aliquot the antibody. Do not freeze.*

Bead Specifications: Releasable Streptavidin Beads are paramagnetic polystyrene beads (4.5 µm diameter) coupled to streptavidin via a DNA linker susceptible to cleavage by DNase I. The beads provided in this kit are not recommended for the isolation of phagocytic cells as the beads are likely to be engulfed by these cells.



Flow cytometric analysis of live cells in the input (left) and post-enrichment (right) for enrichment performed using biotinylated Whitlow/218 Linker (E3U7Q) Rabbit mAb. Input contains a mixture of WT Jurkat cells and Jurkat cells engineered to stably express an scFv-based Anti-CD20 (Leu16) CAR containing a Whitlow/218 linker. Tag Blue fluorescent protein (TagBFP) is co-expressed with the CAR. The post-enrichment sample shows a nearly pure population of cells expressing the CAR on the cell surface. Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 647 Conjugate) #4414 was used as a secondary antibody to detect the biotinylated antibody. Engineered cell line was provided by the Lohmueller Lab, University of Pittsburgh.



Flow cytometric analysis of live cells in the input (left) and post-enrichment (right) for enrichment performed using biotinylated Whitlow/218 Linker (E3U7Q) Rabbit mAb. Input consists of CD4+CD8+ human T cells containing a mixture of non-transduced cells and cells transduced with an scFv-based Anti-BCMA (C11D5.3) CAR containing a Whitlow/218 linker. The post-enrichment sample shows a nearly pure population of cells expressing the CAR on the cell surface. Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 647 Conjugate) #4414 was used as a secondary antibody to detect the biotinylated antibody.

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Applications: W—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **FC-FP**— Flow cytometry-Fixed/Permeabilized **FC-L**— Flow cytometry-Live **E-P**—ELISA-Peptide
Species Cross-Reactivity: H—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse
All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

Cell Enrichment Kit Protocol

NOTE: Kit capacity: ~2 x 10^9 total input cells

A. Solutions and Reagents

Supplied Reagents

- Releasable Streptavidin Beads (1 bottle, 5 mL #27119)
- Lyophilized DNase I (3 vials, > 15,000 units each #91019)
- DNase I Reconstitution Buffer (1 bottle, 2 mL #47776)
- Biotinylated Rabbit mAb (specific to kit)

Additional Reagents (Not Supplied)

NOTE: Refer to Table 1 for recommended tube sizes and volumes.

- Magnet appropriate for tube size
- Mixer allowing tilting and rotation of tubes
- Phosphate Buffered Saline with BSA (PBS-B): Ca^{2+} and Mg^{2+} free PBS supplemented with 0.1% bovine serum albumin (BSA), pH 7.4
- Phosphate Buffered Saline with BSA and EDTA (PBS-BE): Ca^{2+} and Mg^{2+} free PBS with 0.1% BSA (or 0.1% human serum albumin or fetal calf serum) and 2 mM EDTA (or 0.6% sodium citrate)
- Cell Elution Buffer: RPMI 1640 medium with 1% fetal calf serum (FCS), 1 mM $CaCl_2$ and 5 mM $MgCl_2$, pH 7.0–7.4

General Considerations:

- Protocol volumes are suitable for enrichment of 1 x 10^7 total input cells but are directly scalable up to 2 x 10^8 cells. For higher or lower total cell input count, refer to Table 1.
- When enriching for cells expressing targets with high variability of expression, titration of biotinylated mAb and releasable streptavidin beads is recommended to optimize purity and yield.
- Keep all buffers cold unless otherwise specified.
- Avoid air bubbles (foaming) during pipetting.

B. Reagent Preparation

NOTE: For optimal DNase I activity, ensure that the Cell Elution Buffer pH is 7.0-7.4.

NOTE: Reconstitute vials of enzyme as needed.

1. Wash Releasable Streptavidin Beads (#27119):
   - **a.** Resuspend stock vial of beads via vortexing >30 sec or tilt and rotate for 5 min.
   - **b.** Transfer the recommended volume of beads for the number of purifications being performed to a tube (refer to step 5 in Table 1).
   - **c.** Add the same volume, or at least 1 mL, of PBS-B and mix.
   - **d.** Place the tube in a magnet for 1 min, discard the supernatant.
   - **e.** Remove the tube from the magnet and resuspend washed beads in the same volume of PBS-B as the initial volume of beads.
2. Reconstitute DNase I:
   - **a.** To each vial of Lyophilized DNase I #91019 (as needed), add 300 µL of DNase I Reconstitution Buffer #47776.
   - **b.** Dissolve the enzyme gently. Vigorous mixing (e.g., pipetting/vortexing too hard) will destroy its activity.
   - **c.** Aliquot reconstituted DNase I into suitable portions.
   - **d.** Store at -20°C.

     Note: Thaw immediately before use and keep on ice once thawed. Thawed reconstituted DNase I can be refrozen once.
3. Warm appropriate volume of Cell Elution Buffer for the number of purifications being performed to 37°C (refer to step 13 in Table 1).

C. Cell Preparation

NOTE: Blood and serum may contain soluble factors (e.g., antibodies or cell surface antigens) which can interfere with the cell isolation protocol. Washing the cells once may reduce this interference.

NOTE: Optimal centrifugation conditions will vary depending upon cell type and reagent volume. Generally, 180-350 x g for 5-10 min will be sufficient to pellet the cells.

1. Collect cells of interest and pellet by centrifugation.
2. Resuspend cells at 1 x 10^7 cells/mL in PBS-BE at 2-8°C.

D. Cell Enrichment

NOTE: For steps requiring incubations with gentle tilting and rotation, do not perform end-over-end mixing if the volume is small relative to the tube size. Tilt and rotate so the cells and beads are kept in the bottom of the tube.

NOTE: If desired, a small sample of resuspended cells from step 4 can be stained with a fluorochrome-conjugated secondary antibody (e.g., Anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 647 Conjugate) #4414) to determine the percentage of target expressing cells in the input prior to enrichment.

NOTE: Purity of elution fraction can be assessed by flow cytometry using a fluorochrome-conjugated secondary antibody (e.g., Anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 647 Conjugate) #4414).

1. Add 12.5 µL biotinylated antibody to 1 mL cell suspension, mix gently.
2. Incubate for 10 min at 2-8°C.
3. Wash cells by adding 2 mL PBS-BE, then pellet by centrifugation. Discard the supernatant.
4. Resuspend cells at 1 x 10^7 cells/mL in PBS-BE.
5. Resuspend pre-washed beads and add 25 µL to cells.
6. Incubate 20 min at 2-8°C with gentle tilting and rotation.
7. Optional: Add 1 mL PBS-BE to limit trapping of unbound cells.

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8. Place the tube in a magnet for 2 min.
9. While the tube is still in the magnet, remove and discard the supernatant.
10. Remove the tube from the magnet and add 1 mL PBS-B, pipet 2-3 times or vortex 2-3 sec, replace tube in magnet for 2 min.
11. Repeat steps 9 and 10 at least twice to wash the cells. This step is critical to obtain high purity of CAR positive cells.
12. While the tube is still in the magnet, remove and discard the supernatant.
13. Resuspend bead-bound cells in 400 µL pre-warmed (37°C) Cell Elution Buffer.
14. Add 4 µL reconstituted DNase I.
15. Incubate for 15 min at room temperature with gentle tilting and rotation.

    NOTE: Before transferring released cells to new tube in step 17, pre-coat collection tubes with Cell Elution Buffer for at least 5 min.
16. Pipet thoroughly at least 5-10 times to maximize cell release (avoid foaming).
17. Place in magnet for 2 min, then transfer the supernatant with released cells into a tube pre-coated with Cell Elution Buffer.
18. Remove tube from magnet, resuspend bead fraction in 400 µL Cell Elution Buffer, repeat steps 16 and 17 once to collect residual cells.

Table 1. Recommended Volumes for Different Cell Numbers

Cell Enrichment Step	Step Description	Volumes Per 1 x 10^7 Total Input Cells	Volumes Per 2 x 10^8 Total Input Cells
	Recommended tube size	5 mL	50 mL
	Recommended magnet	e.g., Invitrogen DynaMag-5	e.g., Invitrogen DynaMag-50
1*	Biotinylated mAb	12.5 µL	250 µL
1*	Cell volume	1 mL	20 mL
3**	Wash cells (PBS-BE)	2 mL	40 mL
4	Resuspend cells (PBS-BE)	1 mL	20 mL
5***	Add beads	25 µL	500 µL
7	Optional: Increase volume (PBS-BE)	1 mL	20 mL
10-11**	Wash cells (PBS-B)	3 x 1 mL	3 x 20 mL
13	Resuspend cells (Cell Elution Buffer)	400 µL	8 mL
14	Release cells (re-constituted DNase I)	4 µL	80 µL
18	Collect residual cells (Cell Elution Buffer)	400 µL	8 mL

* If total cell input count is lower than 1 x 10^7 cells, adjust biotinylated antibody volume and keep cell density at 1 x 10^7 cells/mL. Wash volumes can be kept the same as for 1 x 10^7 cells.

** Adjust buffer volumes to fit the tube you are using.

*** If the *target* expressing cell population is high (e.g., > 2.5 x 10^6 target cells/mL), increase the amount of beads (maximum double the amount).