Solutions for Evaluation of CAR Expression

Specific detection of CAR expression using target antigens

CAR-T cells

CAR

Antigen

Tumor cells

Content

CD19

BCMA

EGFRVIII

ROR1

GPC3

MSLN

CD22
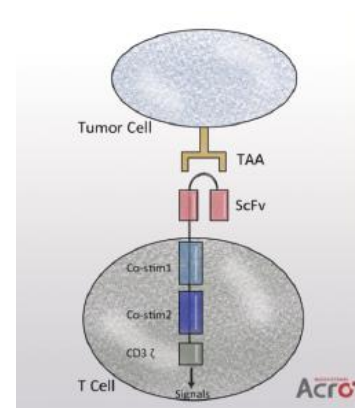
I. Introduction

The chimeric antigen receptor T (CAR-T) cell therapy is a new treatment for a variety of cancers. The idea is to take out the T-cells from the patient, and genetically engineer the cells to make them express a chimeric antigen receptor (CAR) recognizing a specific tumor-associated antigen (TAAs). As a result, the CAR-expressing T cells, when reintroduced into the patient's body, will target and eliminate the TAA-expressing tumor cells.

ACROBiosystems has developed an extensive collection of recombinant proteins to support CAR-T therapy development. This growing list of proteins includes many fluorescent-labeled target antigens and pre-biotinylated proteins that are uniquely suitable for evaluation of CAR expression. In addition, we also supply hard-to-make proteins such as BCMA, CD19, ROR1, and EGFRVIII.

II. Evaluation of CAR Expression

Evaluating CAR expression is an essential step in the production of CAR-T cells. This is often done by flow cytometry, using protein L, anti-Fab antibodies, anti-idiotypic Antibodies or target antigens as detection antibodies. Among these common choices, target antigens are widely considered to be the best option, because it offers high specificity and minimal background staining.

Reagents	Mechanism	Pros	Cons
Target Antigens, Anti-idiotypic Antibodies	Specifically bind to the antigen -binding domains of CARs.	High specificity; Minimal background staining.	Each unique CAR has to be stained with corresponding antigens.
Protein L	Binds to the kappa light chain of immunoglobulin.	Universal.	High background staining; Cannot detect the anti-lambda light chain CAR.
Anti-Fab antibody	Binds to the Fab portion of immunoglobulin.	Universal.	High background staining.

III. CAR Detection Strategy and Product Design

Currently, target antigens for CAR-T cells are most widely used to determine the expression of CARs on gene-modified lymphocytes by flow cytometry. The limitations of these reagents are that many of them are not commercially available. In an effort to fulfill these needs, we have developed an extensive collection of CAR-T target antigens includes many fluorescent-labeled proteins and pre-biotinylated proteins that are uniquely suitable for evaluation of CAR expression by flow cytometry.
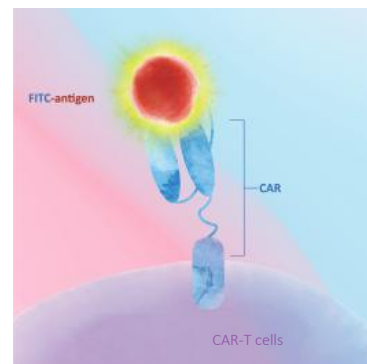
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Detection Methods

■ Direct detection

- Target antigens are pre-labeled with fluorescent dye.
- Processing time can be reduced by the use of direct-labeled proteins.
- Non-specific reaction of a secondary antibody is eliminated.

  ACRO's specially designed products:
 FITC-labeled proteins;
  PE-labeled proteins;

■ Biotin-streptavidin based detection

- Target antigens are pre-labeled with biotin and detected by labeled streptavidin (the biotin-avidin complex).
- Streptavidin labeled with fluorochromes can bind biotinylated proteins with a high degree of affinity and specificity, amplifying the signal and improving the detection sensitivity and specificity.

  ACRO's specially designed products:
  Avitag™ biotinylated proteins;
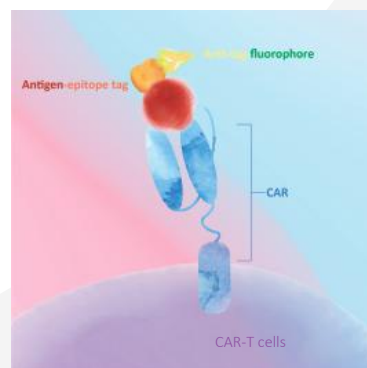 Chemically biotinylated proteins;
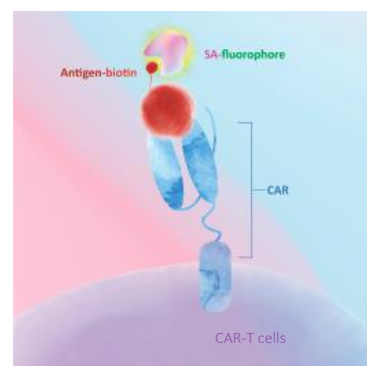
■ Indirect detection

- Target antigens are designed to carry a specific tag and detected using a secondary antibody (anti-epitope tag antibody) labeled with a fluorophore.
- Non-specific reaction of a secondary antibody may occur.

  ACRO's specially designed products:
  Fc-tag fusion proteins;
  His-tag fusion proteins;

FDA DMF Filed Proteins

ACROBiosystems has submitted DMF for its recombinant CD19 and BCMA proteins to FDA, and filed the DMF number as 034936. You can cite this DMF number to shorten the time of preparing the filing of documents to support your IND or BLA.

■ How to get our DMF authorization

If our DMF filed proteins have been used in your drug development process, you can request that we provide DMF authorization to FDA in support of a submission or filing that you have made to the FDA. Please submit your request to ACROBiosystems by leaving your information here. [Request for Authorization]

* If you have FDA DMF filing request for other products, please **contact us**.

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IV. Case Studies

Evaluation of Anti-CD19 CAR Expression with FITC-labeled CD19

■ **Reagents**

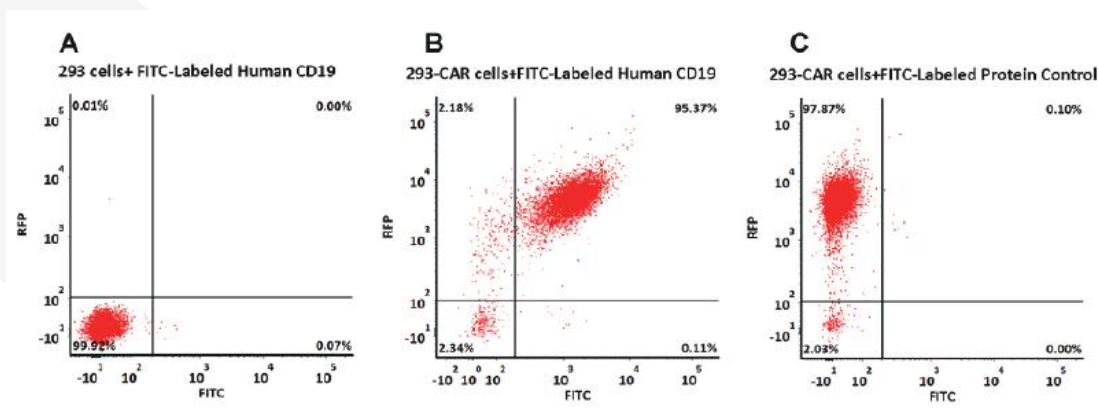FITC-labeled Human CD19 (20-291) Protein (ACROBiosystems, Cat. No. CD9-HF2H2);

■ **Samples**

R1013-C6 cells (Transfected 293 cells expressing the anti-CD19 [FCM63] scFv & RFP tag).

■ **Protocol**

1. Culture R1013-C6 cells in DMEM medium with 10% FBS in the CO_2 incubator (at 37°C, 5% CO_2).
2. Harvest the cells and wash the cells once by wash buffer.
3. Count the cells number and the viability, aliquot up 2e5 live cells (Anti-CD19-scFv positive cell is 98%) into each tube. (Note: the cell viability must be ≥ 95%.)
4. Add 100 μl, 10 μg/ml of FITC-labeled Human CD19 (20-291) Protein or FITC-labeled Protein control into each tube, incubating at 4°C for 1 hour.
5. Wash the cells 3 times by wash buffer and resuspend the cells in 200 μl PBS per sample.
6. Transfer the cells into flow tube and detect by Flow cytometry.
7. Analyze result using FACS Celesta software and FCS Express 6 Flow software.

■ **Results**

The data showed that the expression level of anti-CD19 scFv on the surface of R1013-C6 cells was 95.37% .

293 cells were transfected with anti-CD19-scFv and RFP tag. 2e5 of the cells were stained with B. FITC-Labeled Human CD19 (20-291) (Cat. No. CD9-HF2H2, 10 μg/ml) and C. FITC-labeled protein control. A. Non-transfected 293 cells and C. FITC-labeled protein control were used as negative control. RFP was used to evaluate CAR (anti-CD19-scFv) expression and FITC was used to evaluate the binding activity of FITC-labeled Human CD19 (20-291) (Cat. No. CD9-HF2H2).

The protocol can be provided and feel free to send email to **cart@acrobiosystems.com** to request. Click here

Evaluation of Anti-BCMA CAR Expression with Biotinylated BCMA

■ Reagents

Biotinylated human BCMA protein, Fc & Avi Tag (ACROBiosystems, Cat. No. BC7-H82F0);

■ Samples

Anti-BCMA CAR-transduced human primary T-cells.

■ Brief Protocol

1. Human T cells transfected with anti-BCMA CAR were harvested 3 days after the transfection.

2. Aliquot up to 1e6 cells into centrifuge tube and wash the cells twice with FACS buffer.

3. Resuspend cells in 50 µl of diluted biotinylated human BCMA (ACROBiosystems, Cat. No. BC7-H82F0) (prepared in FACS buffer at 8 µg/ml) and incubate at 4°C for 30 minutes.

4. Wash cells twice with FACS buffer.

5.Resuspend cells in 50 µl of diluted PE Streptavidin (Biolegend, Cat. No. 405204) (prepared in FACS buffer at 1:50 dilution) and incubate at 4°C for 30 minutes in the dark.

6. Wash cells twice with FACS buffer and resuspend the cells in 400 µl PBS.

7. Transfer the cells into flow tube and analyze on BD FACSCalibur™ flow cytometer using FCS Express 6 Plus software.

■ Results

The data showed that the expression of anti-BCMA CARs on transduced T cell surface from donor 1 and donor 2 were 52.72% and 73.49%, respectively.
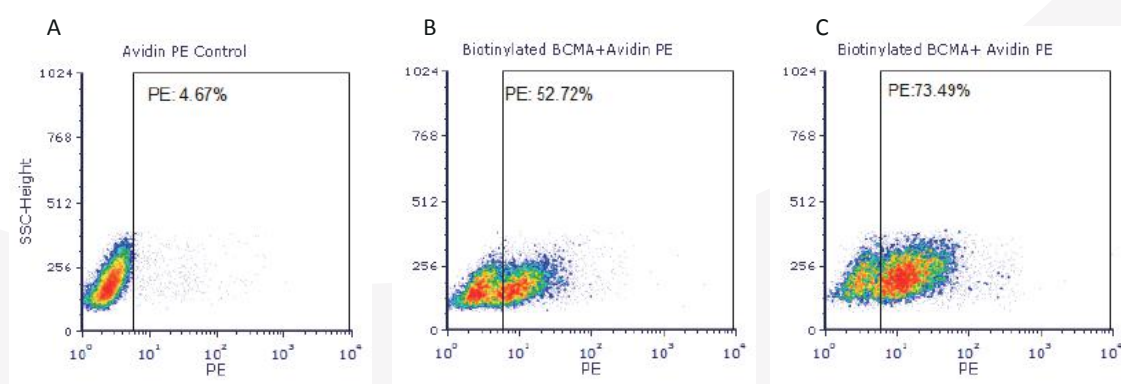
Human T cells were transfected with anti-BCMA CAR and cultured for 3 days. Three days post-transfection, 1e6 cells were first incubated with 50 µl biotinylated human BCMA protein (Cat. No. BC7-H82F0, 8 µg/ml), washed and then stained with PE Streptavidin. (Data are kindly provided by PREGENE Biopharma)

The protocol can be provided and feel free to send email to **cart@acrobiosystems.com** to request.　Click here

Evaluation of Anti-CD19 CAR Expression with FITC-labeled Anti-FMC63 scFv Antibody

Reagents

FITC-Labeled Monoclonal Anti-FMC63 scFv Antibody, Mouse IgG1 (ACROBiosystems, Cat. No. FM3-FY45).

Samples

Anti-CD19 CAR-293 cells

Brief Protocol

1. Culture Anti-CD19 CAR-293 cells in DMEM medium with 10% FBS in the CO_2 incubator (at 37 °C, 5% CO_2).
2. Harvest the cells and wash the cells once by FACS buffer.
3. Count the cells number and the viability, aliquot up 2×10^5 live cells into each tube. (Note: the cell viability must $\geq$ 95%.)
4. Dilute FITC-Labeled Monoclonal Anti-FMC63 scFv Antibody, Mouse IgG1 (ACROBiosystems, Cat. No. FM3-FY45) in FACS buffer to get the working solution just before the assay, and then add 100 µL of the working solution into the tube with cell pellet. Mix well and incubate at 4°C for 60 minutes.
5. Wash the cells 3 times by FACS buffer and resuspend the cell pellet in 200 µL PBS per sample.
6. Transfer the cell suspension into flow tube and detect the cells by Flow cytometry.
7. Analyze the result data using FCS Express 7Plus and GraphPad Prism 5 software.

Results

The data showed that the expression level of anti-CD19 scFv on the surface of anti-CD19 CAR-293 cells was 100%
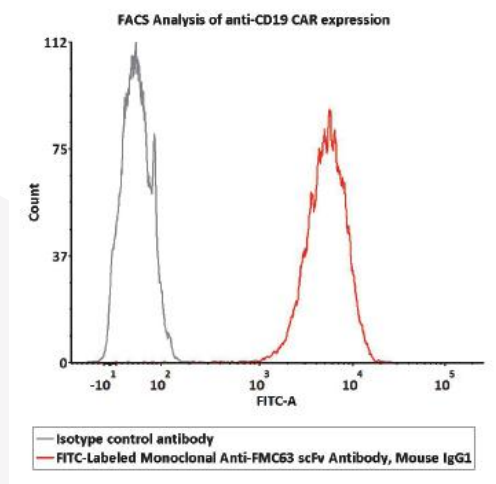
2e5 of Anti-CD19 CAR-293 cells were stained with 100 µL of 1:50 dilution (2 µL stock solution in 100 µL FACS buffer) FITC-Labeled Monoclonal Anti-FMC63 scFv Antibody, Mouse IgG1 (Cat. No. FM3-FY45) and isotype control respectively. FITC signal was used to evaluate the binding activity (QC tested).

The protocol can be provided and feel free to send email to **cart@acrobiosystems.com** to request. Click here

Evaluation of Anti-MSLN CAR Expression with PE-labeled MSLN

■ Reagents

PE-labeled Human Mesothelin / MSLN (296-580) Protein (ACROBiosystems, Cat. No. MSN-HP223);

■ Samples

CAR-RC218 cells (Transfected 293 cells expressing the anti-MSLN scFv).

■ Protocol

1. Culture CAR-RC218 cells in DMEM medium with 10% FBS in the CO_2 incubator (at 37°C, 5% CO_2).
2. Harvest the cells and wash the cells once by wash buffer.
3. Count the cells number and the viability, aliquot up 1e6 live cells into each tube.
4. Add 100 μl of diluted PE-labeled Human Mesothelin (296-580) Protein (Cat. No. MSN-HP223) (prepared in dilution buffer at 1:25 dilution) into each tube, incubating at 4°C for 1 hour.
5. Wash the cells 3 times by wash buffer and resuspend the cells in 200 μl PBS per sample.
6. Transfer the cells into flow tube and detect by Flow cytometry.
7. Analyze result using FACS Celesta software and FCS Express 6 Flow software.

■ Results

The data showed that the expression level of anti-MSLN scFv on the surface of R1013-C6 cells was 100 % .
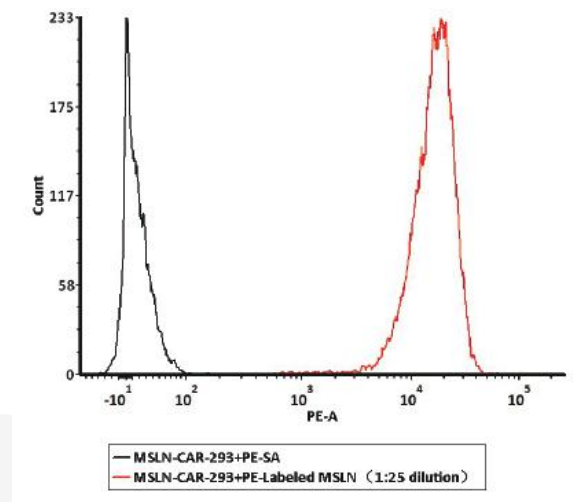
1e6 of the MSLN-CAR-293 cells were stained with 100 μl of 1:25 dilution (4 μl stock solution in 100 μl dilution buffer) of PE- labeled Human Mesothelin / MSLN (296-580) Protein (Cat. No. MSN-HP223). PE Streptavidin was used as negative control (QC tested).

The protocol can be provided and feel free to send email to **cart@acrobiosystems.com** to request. Click here

V. Product List of CAR-T Targets

Fluorescent-labeled Proteins

Targets	Cat. No.	Product Description
BCMA	BCA-HF254	**FITC-labeled** Human BCMA, Fc Tag, **DMF Filed**
BCMA	BCA-HF2H1	**FITC-labeled** Human BCMA, His Tag, **DMF Filed**
CD19	CD9-HF2H2	**FITC-labeled** Human CD19, His Tag, **DMF Filed**
CD19	CD9-HF251	**FITC-labeled** Human CD19, Fc Tag, **DMF Filed**
CD19	CD9-HP2H3	**PE-Labeled** Human CD19 (20-291) Protein, His Tag
CD22	SI2-HF2H6	**FITC-labeled** Human Siglec-2 / CD22, His Tag
CD30	CD0-HF2H3	**FITC-labeled** Human CD30, His Tag
CD4	CD4-HF255	**FITC-labeled** Human CD4, Fc Tag
CD4	CD4-HF2H7	**FITC-labeled** Human CD4, His Tag
EGF R	EGR-HF2H5	**FITC-labeled** EGF R Protein, His Tag
FOLR1	FO1-HF2H8	**FITC-labeled** FOLR1, His Tag
GPC3	GP3-HF258	**FITC-labeled** Human GPC3, Fc Tag
GPC3	GP3-HF2H1	**FITC-labeled** Human GPC3, His Tag
GUCY2C	GUC-HF2H8	**FITC-labeled** Human GUCY2C / Guanylyl cyclase C Protein, His Tag
Her2	HE2-HF224	**FITC-labeled** Human Her2, His Tag
Her3	ER3-HF2H5	**FITC-labeled** Human ErbB3, His Tag
IL13RA2	IL2-HF2H3	**FITC-labeled** Human IL-13 R alpha 2 Protein, His Tag
MSLN	MSN-HF253	**FITC-labeled** Human MSLN, Fc Tag
MSLN	MSN-HF223	**FITC-Labeled** Human MSLN, His Tag
MSLN	MSN-HP223	**PE-labeled** Human MSLN, His Tag
MSLN	MSN-HG2P4	**GFP** Fusion Human Mesothelin / MSLN (296-580) Protein, His Tag

Fluorescent-labeled Proteins

Targets	Cat. No.	Product Description
MSLN	MSN-HR2P4	**RFP** Fusion Human Mesothelin / MSLN (296-580) Protein, His Tag
MSLN	MSN-HF25x	**FITC-labeled** Human Mesothelin / MSLN (296-580) Protein, Fc Tag
PSMA	PSA-HF244	**FITC-labeled** Human PSMA, His Tag
SLAMF7	SL7-HF2H7	**FITC-labeled** Human SLAMF7, His Tag

Biotinylated Proteins

Targets	Cat. No.	Product Description
BCMA	BC7-H82F0	Biotinylated Human BCMA, Fc & Avi Tag, **DMF Filed**
BCMA	BCA-H82E4	Biotinylated Human BCMA, His & Avi Tag, **DMF Filed**
CD147	CD7-H82E0	Biotinylated Human EMMPRIN / CD147 Protein, Avi & His Tag
CD19	CD9-H8259	Biotinylated Human CD19, Fc Tag, **DMF Filed**
CD19	CD9-H82E9	Biotinylated Human CD19 (20-291) Protein, His,Avitag™ (SPR verified)
CD19	CD9-H82F7	Biotinylated Human CD19 (20-291) Protein, Fc,Avitag™
CD22	SI2-H82F8	Biotinylated Human CD22, Fc & Avi Tag
CD30	CD0-H82E6	Biotinylated Human CD30, Avi & His Tag
CD33	CD3-H82E7	Biotinylated Human CD33, Avi & His Tag
CD38	CD8-H82E7	Biotinylated Human CD38, Avi & His Tag
CD4	CD4-H82F3	Biotinylated Human CD4, Fc & Avitag
CD4	CD4-H82E8	Biotinylated Human CD4, His & Avitag
CD70	TN7-H82F4	Biotinylated Human CD70, Avi & Fc Tag
CEACAM5	CE5-H82E0	Biotinylated Human CEACAM-5, His & Avitag
EGF R	EGR-H82E3	Biotinylated Human EGFR, His & Avi Tag

Biotinylated Proteins

Targets	Cat. No.	Product Description
EpCAM	EPM-H8223	Biotinylated Human EpCAM, His Tag
EpCAM	EPM-H8254	Biotinylated Human EpCAM, Fc Tag
EpCAM	EPM-H82E8	Biotinylated Human EpCAM, Avi & His Tag
EpCAM	EPM-H82F9	Biotinylated Human EpCAM, Fc & Avi Tag
FOLR1	FO1-H82E2	Biotinylated Human FOLR1, His & Avi Tag
FOLR1	FO1-H82F9	Biotinylated Human FOLR1, Fc & Avi Tag
GPC3	GP3-H82E5	Biotinylated Human GPC3, His & Avi Tag
Her2	HE2-H822R	Biotinylated Human Her2, His Tag
Her2	HE2-H82E2	Biotinylated Human Her2, His & Avi Tag
Her3	ER3-H8223	Biotinylated Human ErbB3, His Tag
Her3	ER3-H82E6	Biotinylated Human ErbB3, His & Avitag
HGF R	MET-H82E1	Biotinylated Human HGF R, Avi & His Tag
MSLN	MSN-H8223	Biotinylated Human MSLN, His Tag
MSLN	MSN-H826x	Biotinylated Human MSLN, Fc Tag
MSLN	MSN-H82E9	Biotinylated Human MSLN, His & Avi Tag
MSLN	MSN-H82F6	Biotinylated Human MSLN, Fc & Avi Tag
MUC16	CA5-H82F4	Biotinylated Human CA125 / MUC16 Protein, Fc & Avi Tag
Nectin-4	NE4-H82E7	Biotinylated Human Nectin-4, His & Avi Tag
Protein L	RPL-P814R	Biotinylated Recombinant Protein L, His Tag
ROR1	RO1-H82E6	Biotinylated Human ROR1, His & Avi Tag
ROR1	RO1-H82F4	Biotinylated Human ROR1, Fc & Avi Tag
SLAMF7	SL7-H82E0	Biotinylated Human SLAMF7 / CRACC / CD319 Protein, His Tag, Avi Tag
VEGFR2	KDR-H82E5	Biotinylated Human VEGF R2, Avi & His Tag

Unconjugated Proteins

Targets	Cat. No.	Product Description
BCMA	BC7-H5254	Human BCMA, Fc Tag, **DMF Filed**
BCMA	BCA-H5259	Human BCMA, Llama IgG2b Fc Tag, low endotoxin
BCMA	BCA-H522y	Human BCMA, His Tag, **DMF Filed**
BCMA	BCA-H5253	Human BCMA, Mouse IgG2a Fc Tag, low endotoxin
CAIX	CA9-H5226	Human Carbonic Anhydrase IX / CA9, His Tag
CD133	CD3-H55H7	Human CD133, His Tag (insect cell)
CD19	CD9-H52H2	Human CD19, His Tag, **DMF Filed**
CD19	CD9-H5250	Human CD19, Llama IgG2b Fc Tag, low endotoxin
CD19	CD9-H5251	Human CD19, Fc Tag, low endotoxin (Super affinity), **DMF Filed**
CD19	CD9-H5258	Human CD19 (20-291) Protein, Mouse IgG2a Fc Tag
CD70	TN7-H526x	Human CD27 Ligand / CD70, Fc Tag
CD70	CDL-H525a	Human CD27 Ligand / CD70, Mouse IgG2a Fc Tag, low endotoxin
CD30	CD0-H5229	Human CD30 / TNFRSF8, His Tag
CD30	CD0-H5250	Human CD30 / TNFRSF8, Fc Tag
CD30	TN8-H5250	Human CD30 / TNFRSF8, Llama IgG2b Fc Tag, low endotoxin
CD38	CD8-H5224	Human CD38, His Tag
CD38	CD8-H5255	Human CD38, Fc Tag
CD38	CD8-H5253	Human CD38, Mouse IgG2a Fc Tag, low endotoxin
CD38	CD8-H5252	Human CD38, Llama IgG2b Fc Tag, low endotoxin
CD4	CD4-H5259	Human CD4 (Lys 26 - Pro 396), Fc Tag
CD4	LE3-H5228	Human CD4 (Lys 26 - Trp 390), His Tag
CD7	CD7-H5258	Human CD7, Llama IgG2b Fc Tag

Unconjugated Proteins

Targets	Cat. No.	Product Description
CEACAM5	CE5-H5226	Human CEACAM-5 / CD66e Protein, His Tag
CLL-1	CLA-H5266	Human CLEC12A / MICL / CLL-1 Protein, Fc Tag
CLL-1	CLA-H5245	Human CLEC12A / MICL / CLL-1 Protein, His Tag
EGF R	EGR-H5252	Human EGF R, Fc Tag
EGF R	EGR-H5285	Human EGF R, Strep Tag
EGF R	EGR-H5222	Human EGF R, His Tag
EGF R	EGR-H522a	Human EGF R, His Tag, low endotoxin
CD147	CD7-H5222	Human EMMPRIN / CD147 Protein, His Tag
CD147	CD7-H5259	Human EMMPRIN / CD147 Protein, Fc Tag
EpCAM	EPM-H5223	Human EpCAM / TROP1, His Tag
EpCAM	EPM-H5254	Human EpCAM / TROP1, Fc Tag
FAP	FAP-H5263	Human FAP Protein, Fc Tag
Her3	ER3-H5259	Human ErbB3 / Her3 Protein, Fc Tag
Her3	ER3-H5288	Human ErbB3 / Her3 Protein, Strep Tag
Her3	ER3-H5223	Human ErbB3, His Tag
Her3	ER3-H5257	Human ErbB3 / Her3 Protein, Mouse IgG2a Fc Tag
FOLR1	FO1-H528b	Human FOLR1, Strep Tag
FOLR1	FO1-H5253	Human FOLR1, Fc Tag
FOLR1	FO1-H52H1	Human FOLR1 Protein, His Tag (HPLC-verified)
GPC3	GP3-H5258	Human GPC3, Fc Tag, low endotoxin
GPC3	GP3-H52H4	Human GPC3, His Tag
GUCY2C	GUC-H5257	Human Human GUCY2C, Fc Tag

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Unconjugated Proteins

Targets	Cat. No.	Product Description
GUCY2C	GUC-H52H5	Human GUCY2C, His Tag
Her2	HE2-H5287	Human Her2 / ErbB2, Strep Tag
Her2	HE2-H5225	Human Her2 / ErbB2, His Tag
Her2	HE2-H5253	Human Her2 / ErbB2, Fc Tag
HGF R	MET-H5227	Human HGF R / c-MET, His Tag
HGF R	MET-H5256	Human HGF R / c-MET, Fc Tag
IL13RA2	IL2-H5256	Human IL-13 R alpha 2 Protein, Fc Tag (MALS verified)
CD123	ILA-H52H6	Human IL-3 R alpha / CD123, His Tag
CD123	ILA-H5255	Human IL-3 R alpha / CD123, Llama IgG2b Fc Tag, low endotoxin
MSLN	MSN-H5223	Human MSLN (296-580), His Tag
MSLN	MSN-H5253	Human MSLN (296-580), Fc Tag
MSLN	MSN-H522a	Human MSLN (296-580), His Tag, low endotoxin
MUC1	MU1-H5252	Human Mucin-1 / MUC-1 (33-167), Fc Tag
Nectin-4	NE4-H52H3	Human Nectin-4, His Tag
NKG2D	NKD-H5265	Human NKG2D, Fc Tag
PSCA	PSA-H52H6	Human PSCA Protein, His Tag
PSMA	PSA-H52H3	Human PSMA, His Tag
ROR1	RO1-H5222	Human ROR1 (165-305, Frizzled domain), His Tag
ROR1	RO1-H5221	Human ROR1 (39-151, Ig-like domain), His Tag
ROR1	RO1-H5223	Human ROR1 (308-395, Kringle domain), His Tag
ROR1	RO1-H5250	Human ROR1 (30-403), Fc Tag
ROR1	RO1-H522y	Human ROR1 (30-403), His Tag

Unconjugated Proteins

Targets	Cat. No.	Product Description
CD22	SI2-H5228	Human Siglec-2 / CD22 (176-687), His Tag
CD22	CD2-H52H8	Human Siglec-2 / CD22 (20-687), His Tag
CD22	SI2-H525a	Human Siglec-2 / CD22 (20-687), Llama IgG2b Fc Tag, low endotoxin
CD33	CD3-H5226	Human Siglec-3 / CD33, His Tag
CD33	CD3-H5257	Human Siglec-3 / CD33, Fc Tag
CD33	CD3-H5259	Human Siglec-3 / CD33, Llama IgG2b Fc Tag, low endotoxin
SLAMF7	SL7-H5225	Human SLAMF7 / CRACC / CD319 Protein
SLAMF7	SL7-H5256	Human SLAMF7 / CRACC / CD319 Protein, Fc Tag
CD138	SD1-H5228	Human Syndecan-1 / CD138, His Tag
VEGFR2	KDR-H5280	Human VEGF R2 / KDR Protein, Strep Tag
VEGFR2	KDR-H5227	Human VEGF R2, His Tag (HPLC-verified)