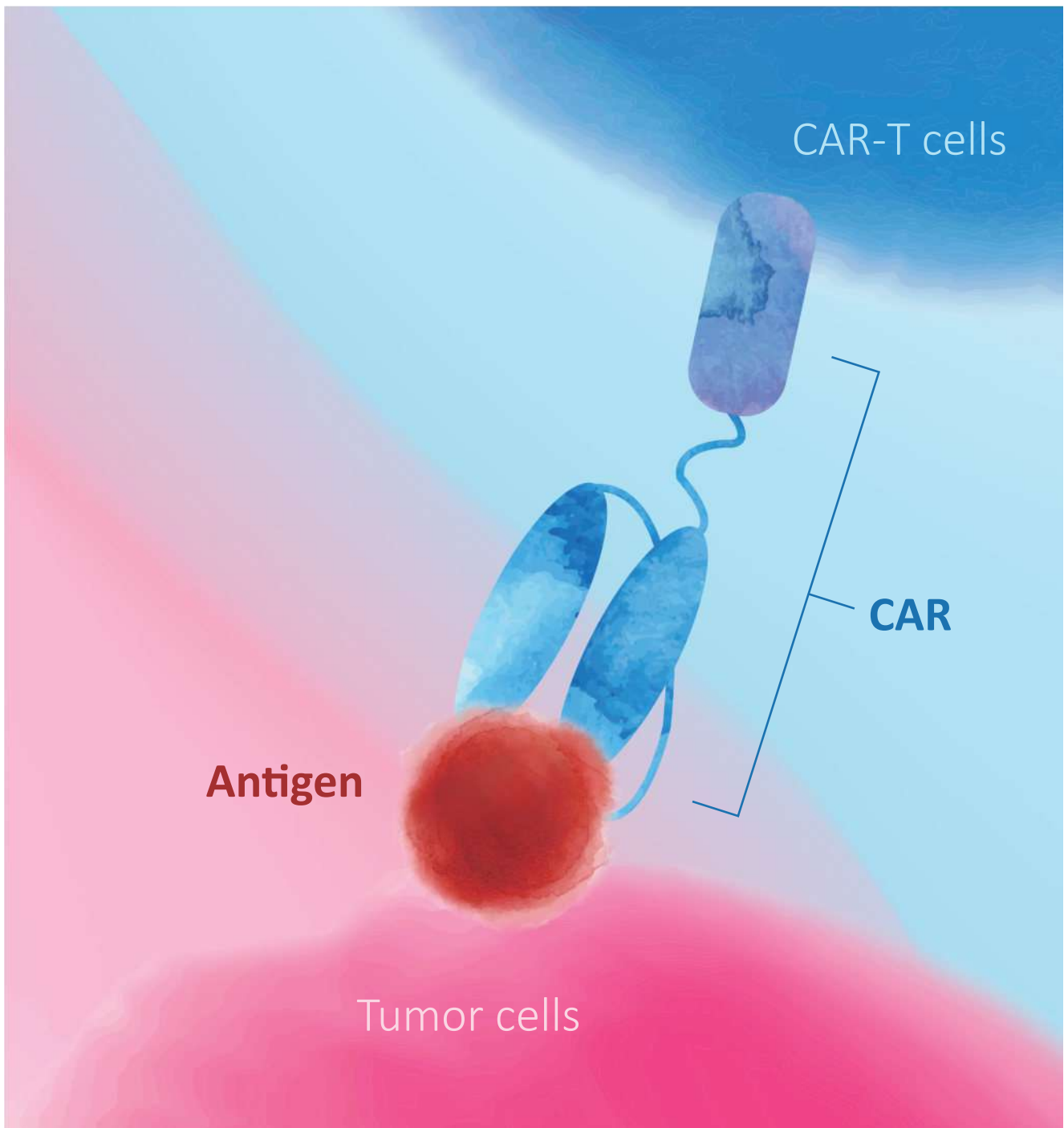


Solutions for Evaluation of CAR Expression

Specific detection of CAR expression using target antigens



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ROR1
CD22

MSLN

GPC3

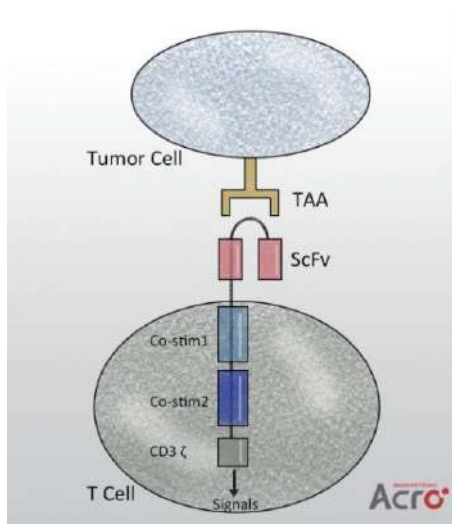
BCMA

CD19
EGFRVIII

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.

2017 is a milestone year for CAR-T cell therapy. The FDA approved two CD19-targeting CAR-T therapies. Novartis's Kymriah is approved for B-cell precursor ALL treatment in children and young adults, while Kite Pharma's Yescarta is approved for the treatment of adult patients. This success fueled the efforts to develop / advance CAR-T treatment targeting other cancer-specific antigens. Beyond CD19, there is a growing list of targets being investigated for therapeutic intervention.



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 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	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.

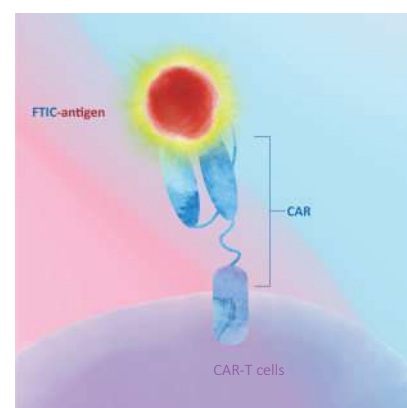
Detection Methods

■ 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;

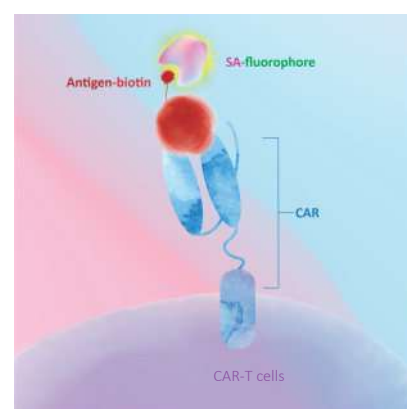


■ Biotin-streptavidin based detection

- 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;

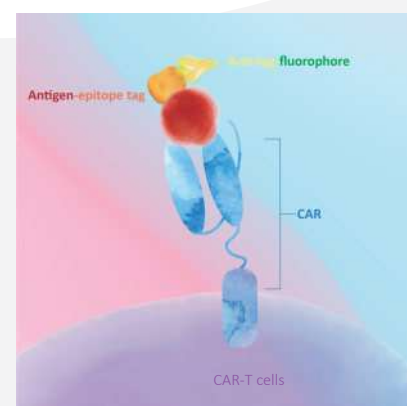


■ Indirect detection

- 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;



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

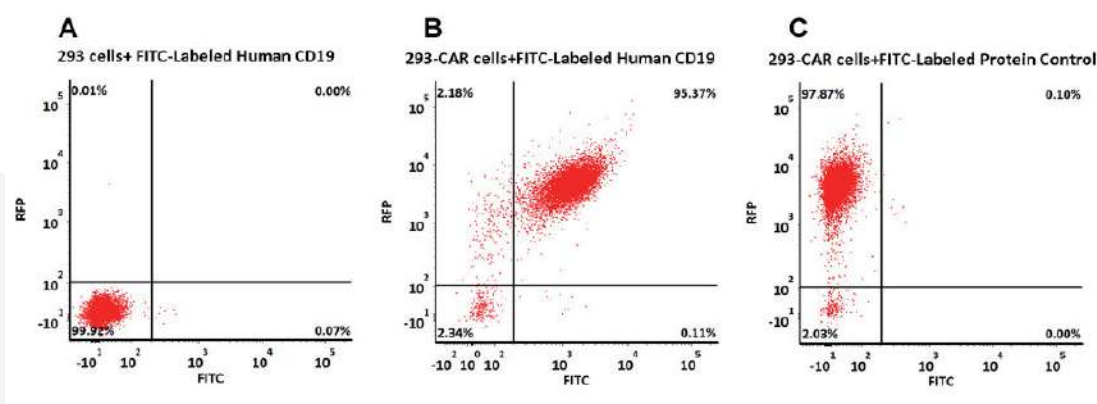
R1013-C6 cells (Transfected 293 cells expressing the anti-CD19 [FCM63] scFv & RFP tag).

■ Protocol

1. Culture R1013-C6 cells in DMEM medium with 10% FBS in the CO₂ incubator (at 37°C, 5%CO₂).
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 $\geq 95\%$.)
4. Add 100 μ l, 10 μ g/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 μ l 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 μ g/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).

Please feel free to contact us by cart@acrobiosystems.com if you have any questions or require detailed protocols.

Evaluation of Anti-BCMA CAR Expression with Biotinylated BCMA

■ Reagents

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

■ Samples

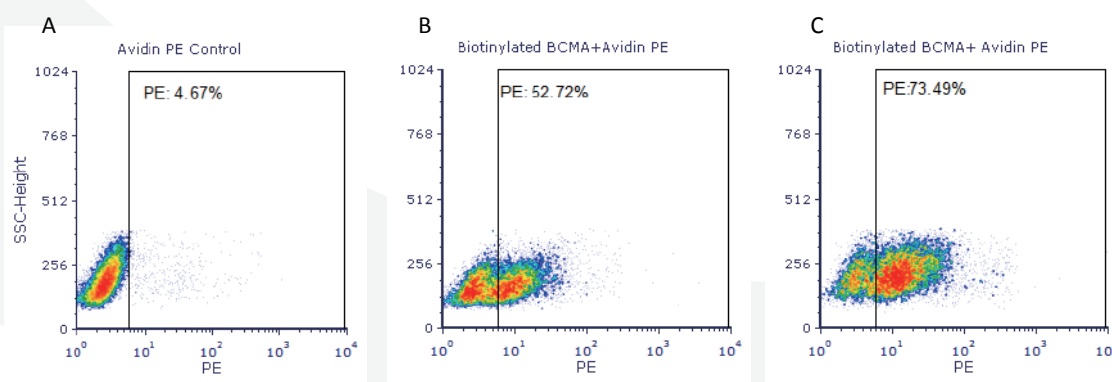
Anti-BCMA CAR-transduced human primary T-cells.

■ 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 μ l of diluted biotinylated human BCMA (ACROBiosystems, Cat. No. BC7-H82F0) (prepared in FACS buffer at 8 μ g/ml) and incubate at 4°C for 30 minutes.
4. Wash cells twice with FACS buffer.
5. Resuspend cells in 50 μ l 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 μ l 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 μ l biotinylated human BCMA protein (Cat. No. BC7-H82F0, 8 μ g/ml), washed and then stained with PE Streptavidin. (Data are kindly provided by PREGENE Biopharma)

Please feel free to contact us by cart@acrobiosystems.com if you have any questions or require detailed protocols.

Evaluation of Anti-CD19 CAR Expression with Fc-fusion CD19

■ Reagents

Human CD19 protein, Fc Tag (Acrobiosystems, Cat. No. CD9-H5251);
FITC anti-human IgG Fc antibody (Biolegend, Cat. No. 409310).

■ Samples

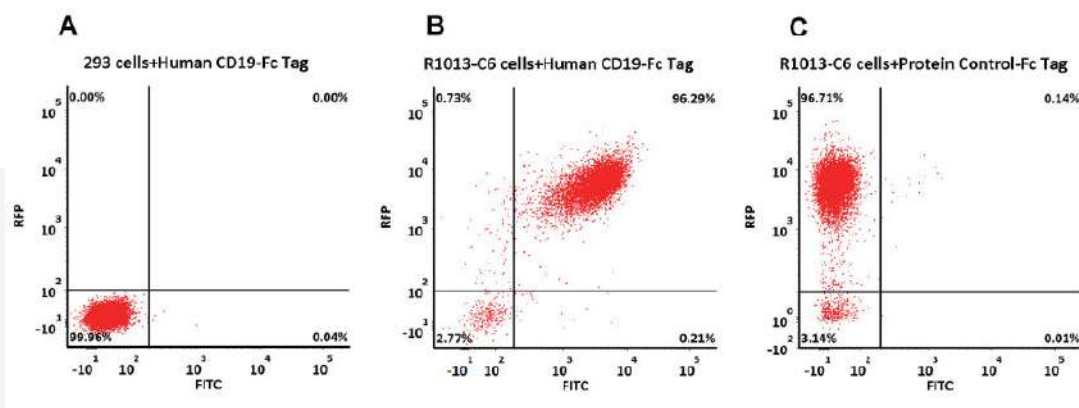
R1013-C6 cells (Transfected 293 cells expressing the anti-CD19 [FCM63] scFv & RFP tag).

■ Brief Protocol

1. Culture R1013-C6 cells in DMEM medium with 10% FBS in the CO₂ incubator (at 37°C, 5%CO₂).
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 \geq 95%.)
4. Add 100 μ l, 3 μ g/ml of Recombinant Human CD19 (20-291) Protein, Fc Tag or Recombinant Human Fc Tag Protein control into each tube, incubating at 4°C for 1 hour.
5. Wash the cells once by wash buffer.
6. Add FITC anti-human IgG Fc (Biolegend, Cat. No. 409310) by dilution of 1:100 (100 μ l), incubating at 4°C for 1 hour.
7. Wash the cells 3 times by wash buffer and resuspend the cells in 200 μ l PBS per sample.
8. Transfer the cells into flow tube and detect by Flow cytometry.
9. 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 96.29%.



293 cells were transfected with FCM63-scFv and RFP tag. 2e5 of the cells were first stained with B. Human CD19 (20-291) Protein, Fc Tag, low endotoxin (Super affinity) (Cat. No. CD9-H5251, 3 μ g/ml) and C. Human Fc Tag Protein Control, followed by FITC-conjugated Anti-human IgG Fc Antibody. A. Non-transfected 293 cells and C. Human Fc Tag 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 Human CD19 (20-291) Protein, Fc Tag.

Please feel free to contact us by cart@acrobiosystems.com if you have any questions or require detailed protocols.

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

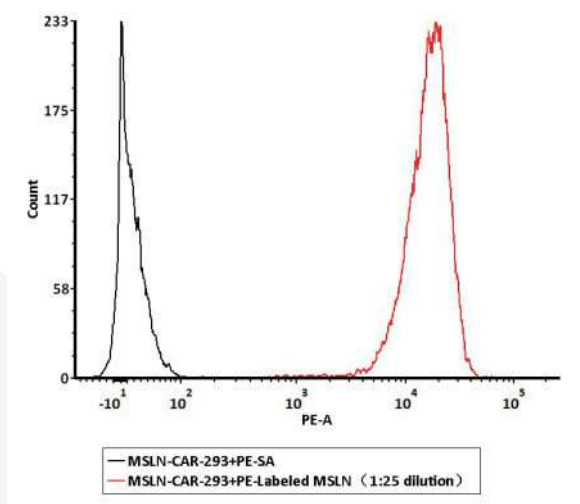
CAR-RC218 cells (Transfected 293 cells expressing the anti-MSLN scFv).

■ Protocol

1. Culture CAR-RC218 cells in DMEM medium with 10% FBS in the CO₂ incubator (at 37°C, 5%CO₂).
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).

Please feel free to contact us by cart@acrobiosystems.com if you have any questions or require detailed protocols.

V. Product List of CAR-T Targets

Fluorescent-labeled Proteins

Targets	Cat. No.	Product Description	Diseases
BCMA	BCA-HF2H1	FITC-labeled Human BCMA, His Tag	Multiple myeloma, leukemia, B-Cell lymphoma
BCMA	BCA-HF254	FITC-labeled Human BCMA, Fc Tag	Multiple myeloma, leukemia, B-Cell lymphoma
CD19	CD9-HF2H2	FITC-labeled Human CD19, His Tag	Acute leukemia, B-Cell lymphoma
CD19	CD9-HF251	FITC-labeled Human CD19, Fc Tag	Acute leukemia, B-Cell lymphoma
CD22	SI2-HF2H6	FITC-labeled Human Siglec-2 / CD22, His Tag	Leukemia, B-Cell lymphoma
Glypican 3	GP3-HF258	FITC-labeled Human GPC3, Fc Tag	Hepatocellular carcinoma
Mesothelin	MSN-HF223	FITC-labeled Human MSLN, His Tag	Mesothelioma, ovarian cancer
Mesothelin	MSN-HF253	FITC-labeled Human MSLN, Fc Tag	Mesothelioma, ovarian cancer
Mesothelin	MSN-HP223	PE-labeled Human MSLN, His Tag	Mesothelioma, ovarian cancer
Protein L	RPL-PF141	FITC-labeled Recombinant Protein L	--

Biotinylated Proteins

Targets	Cat. No.	Product Description	Diseases
BCMA	BCA-H82E4	Biotinylated Human BCMA, His & Avi Tag	Multiple myeloma, leukemia, B-Cell lymphoma
BCMA	BC7-H82F0	Biotinylated Human BCMA, Fc & Avi Tag	Multiple myeloma, leukemia, B-Cell lymphoma
CD19	CD9-H8259	Biotinylated Human CD19, Fc Tag	Acute leukemia, B-Cell lymphoma
CD22	SI2-H82F8	Biotinylated Human CD22, Fc & Avi Tag	Leukemia, B-Cell lymphoma
EGFRvIII	EGR-H82E0	Biotinylated Human EGFRvIII, Avi & His Tag	Glioblastoma
Glypican 3	GP3-H82E5	Biotinylated Human GPC3, His & Avi Tag	Hepatocellular carcinoma
Mesothelin	MSN-H82E9	Biotinylated Human MSLN, His & Avi Tag	Mesothelioma, ovarian cancer
Mesothelin	MSN-H8223	Biotinylated Human MSLN, His Tag	Mesothelioma, ovarian cancer



Biotinylated Proteins

Targets	Cat. No.	Product Description	Diseases
Mesothelin	MSN-H82F6	Biotinylated Human MSLN, Fc & Avi Tag	Mesothelioma, ovarian cancer
Mesothelin	MSN-H826x	Biotinylated Human MSLN, Fc Tag	Mesothelioma, ovarian cancer
CD30	CD0-H82E6	Biotinylated Human CD30, Avi & His Tag	Leukemia, B-Cell lymphoma
CD33	CD3-H82E7	Biotinylated Human CD33, Avi & His Tag	Acute myeloid leukemia
CD38	CD8-H82E7	Biotinylated Human CD38, Avi & His Tag	B-cell Malignancies
CD70	CDL-H82Q9	Biotinylated Human CD70, His & Avi Tag	Glioblastoma
CD70	TN7-H82F4	Biotinylated Human CD70, Avi & Fc Tag	Glioblastoma
EGF R	EGR-H82E3	Biotinylated Human EGFR, His & Avi Tag	NSCLC, epithelial carcinoma, glioma
EpCAM	EPM-H82E8	Biotinylated Human EpCAM, Avi & His Tag	Liver neoplasms, stomach neoplasms
EpCAM	EPM-H8223	Biotinylated Human EpCAM, His Tag	Liver neoplasms, stomach neoplasms
EpCAM	EPM-H82F9	Biotinylated Human EpCAM, Fc & Avi Tag	Liver neoplasms, stomach neoplasms
EpCAM	EPM-H8254	Biotinylated Human EpCAM, Fc Tag	Liver neoplasms, stomach neoplasms
FOLR1	FO1-H82E2	Biotinylated Human FOLR1, His & Avi Tag	Ovarian cancer
FOLR1	FO1-H82F9	Biotinylated Human FOLR1, Fc & Avi Tag	Ovarian cancer
HER2	HE2-H82E2	Biotinylated Human Her2, His & Avi Tag	Ovarian cancer, breast cancer, glioblastoma, osteosarcoma
HER2	HE2-H822R	Biotinylated Human Her2, His Tag	Ovarian cancer, breast cancer, glioblastoma, osteosarcoma
HGF R	MET-H82E1	Biotinylated Human HGF R, Avi & His Tag	Malignant melanoma, breast cancer
ROR1	RO1-H82E6	Biotinylated Human ROR1, His & Avi Tag	Leukemia, breast cancer
ROR1	RO1-H82F4	Biotinylated Human ROR1, Fc & Avi Tag	Leukemia, breast cancer
Protein L	RPL-P814R	Biotinylated Recombinant Protein L, His Tag	--

Unconjugated Proteins

Targets	Cat. No.	Product Description	Diseases
BCMA	BCA-H522y	Human BCMA, His Tag	Multiple myeloma, leukemia, B-Cell lymphoma
BCMA	BC7-H5254	Human BCMA, Fc Tag	Multiple myeloma, leukemia, B-Cell lymphoma
BCMA	BCA-H5253	Human BCMA, Mouse IgG2a Fc Tag, low endotoxin	Multiple myeloma, leukemia, B-Cell lymphoma
BCMA	BCA-H5259	Human BCMA, Llama IgG2b Fc Tag, low endotoxin	Multiple myeloma, leukemia, B-Cell lymphoma
CD19	CD9-H52H2	Human CD19, His Tag	Acute leukemia, B-Cell lymphoma
CD19	CD9-H5251	Human CD19, Fc Tag, low endotoxin (Super affinity)	Acute leukemia, B-Cell lymphoma
CD19	CD9-H5250	Human CD19, Llama IgG2b Fc Tag, low endotoxin	Acute leukemia, B-Cell lymphoma
CD123	ILA-H52H6	Human IL-3 R alpha / CD123, His Tag	Acute Myeloid Leukemia
CD123	ILA-H5255	Human IL-3 R alpha / CD123, Llama IgG2b Fc Tag, low endotoxin	Acute Myeloid Leukemia
CD138	SD1-H5228	Human Syndecan-1 / CD138, His Tag	Multiple myeloma
CD22	SI2-H5228	Human Siglec-2 / CD22 (176-687), His Tag	Leukemia, B-Cell lymphoma
CD22	CD2-H52H8	Human Siglec-2 / CD22 (20-687), His Tag	Leukemia, B-Cell lymphoma
CD22	SI2-H525a	Human Siglec-2 / CD22 (20-687), Llama IgG2b Fc Tag, low endotoxin	Leukemia, B-Cell lymphoma
CD30	CD0-H5229	Human CD30 / TNFRSF8, His Tag	Leukemia, B-Cell lymphoma
CD30	CD0-H5250	Human CD30 / TNFRSF8, Fc Tag	Leukemia, B-Cell lymphoma
CD30	TN8-H5250	Human CD30 / TNFRSF8, Llama IgG2b Fc Tag, low endotoxin	Leukemia, B-Cell lymphoma
CD33	CD3-H5226	Human Siglec-3 / CD33, His Tag	Acute myeloid leukemia
CD33	CD3-H5257	Human Siglec-3 / CD33, Fc Tag	Acute myeloid leukemia
CD33	CD3-H5259	Human Siglec-3 / CD33, Llama IgG2b Fc Tag, low endotoxin	Acute myeloid leukemia
CD38	CD8-H5224	Human CD38, His Tag	B-cell Malignancies
CD38	CD8-H5255	Human CD38, Fc Tag	B-cell Malignancies
CD38	CD8-H5253	Human CD38, Mouse IgG2a Fc Tag, low endotoxin	B-cell Malignancies



Unconjugated Proteins

Targets	Cat. No.	Product Description	Diseases
CD38	CD8-H5252	Human CD38, Llama IgG2b Fc Tag, low endotoxin	B-cell Malignancies
CD70	TN7-H526x	Human CD27 Ligand / CD70, Fc Tag	Glioblastoma
CD70	CDL-H525a	Human CD27 Ligand / CD70, Mouse IgG2a Fc Tag, low endotoxin	Glioblastoma
CAIX	CA9-H5226	Human Carbonic Anhydrase IX / CA9, His Tag	Renal cell carcinoma (RCC)
EGF R	EGR-H5222	Human EGF R, His Tag	NSCLC, epithelial carcinoma, glioma
EGF R	EGR-H522a	Human EGF R, His Tag, low endotoxin	NSCLC, epithelial carcinoma, glioma
EGF R	EGR-H5252	Human EGF R, Fc Tag	NSCLC, epithelial carcinoma, glioma
EGF R	EGR-H5285	Human EGF R, Strep Tag	NSCLC, epithelial carcinoma, glioma
EGFRVIII	EGI-H52H4	Human EGFRVIII, His Tag	Glioblastoma
EpCAM	EPM-H5223	Human EpCAM / TROP1, His Tag	Liver neoplasms, stomach neoplasms
EpCAM	EPM-H5254	Human EpCAM / TROP1, Fc Tag	Liver neoplasms, stomach neoplasms
EpCAM	EPM-H5283	Human EpCAM / TROP1, Strep Tag	Liver neoplasms, stomach neoplasms
FOLR1	FO1-H5229	Human FOLR1, His Tag	Ovarian cancer
FOLR1	FO1-H5253	Human FOLR1, Fc Tag	Ovarian cancer
FOLR1	FO1-H528b	Human FOLR1, Strep Tag	Ovarian cancer
GPC3	GP3-H52H4	Human GPC3, His Tag	Hepatocellular carcinoma
GPC3	GP3-H5258	Human GPC3, His Tag	Hepatocellular carcinoma
HER2	HE2-H5225	Human Her2 / ErbB2, His Tag	Ovarian cancer, breast cancer, glioblastoma, osteosarcoma
HER2	HE2-H5253	Human Her2 / ErbB2, Fc Tag	Ovarian cancer, breast cancer, glioblastoma, osteosarcoma
HER2	HE2-H5287	Human Her2 / ErbB2, Strep Tag	Ovarian cancer, breast cancer, glioblastoma, osteosarcoma
HGF R	MET-H5227	Human HGF R / c-MET, His Tag	Malignant melanoma, breast cancer
HGF R	MET-H5256	Human HGF R / c-MET, Fc Tag	Malignant melanoma, breast cancer

Unconjugated Proteins

Targets	Cat. No.	Product Description	Diseases
IL13RA2	IL2-H5257	Human IL-13 R alpha 2, Fc Tag	Glioma
Mesothelin	MSN-H5223	Human MSLN (296-580), His Tag	Mesothelioma, ovarian cancer
Mesothelin	MSN-H522a	Human MSLN (296-580), His Tag, low endotoxin	Mesothelioma, ovarian cancer
Mesothelin	MSN-H5253	Human MSLN (296-580), Fc Tag	Mesothelioma, ovarian cancer
MUC1	MU1-H5252	Human Mucin-1 / MUC-1 (33-167), Fc Tag	Seminal vesicle cancer
ROR1	RO1-H522y	Human ROR1 (30-403), His Tag	Leukemia, breast cancer
ROR1	RO1-H5250	Human ROR1 (30-403), Fc Tag	Leukemia, breast cancer
ROR1	RO1-H5222	Human ROR1 (165-305, Frizzled domain), His Tag	Leukemia, breast cancer
ROR1	RO1-H5221	Human ROR1 (39-151, Ig-like domain), His Tag	Leukemia, breast cancer
ROR1	RO1-H5223	Human ROR1 (308-395, Kringle domain), His Tag	Leukemia, breast cancer

TIM-3 CTLA-4 4-1BB
Immune Checkpoint
Biotinylated HER2
B7-H4 TIGIT 4-1BB Ligand
FCRn CD40 GITR
PD-L2 HER2
DNAM-1 B7-1
TIM-3 FcRn
TNF- alpha LAG-3
CD19 OX40 Ligand B7-H2
PD-1 CD47 PCSK9
Immune Checkpoint
Biotin-labeled
VEGF165
FcRn HER2 BTLA ICOS CD27 CD48
B7-H4 TIM-3 CTLA-4
Biotin-labeled
HER2 PD-L2 B7-H4 DNAM-1
VEGF165 FcRn PD-L1
BTTLA CTLA-4
DNAM-1 PD-L2 FcRn
PD-L2 PD-L2
GTR Ligand Biotinylated PD-1
TNF-alpha PD-L2