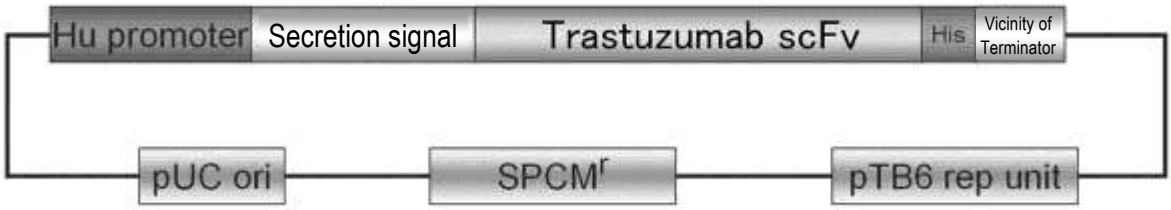
[Figure 1]

Structure of Trastuzumab scFv secretion plasmid



Vector framework: pBEshuttle (having SPCM^r and replication origins of E. coli and bifidobacteria)

[Figure 2]

Total nucleotide sequence of Trastuzumab scFv antibody

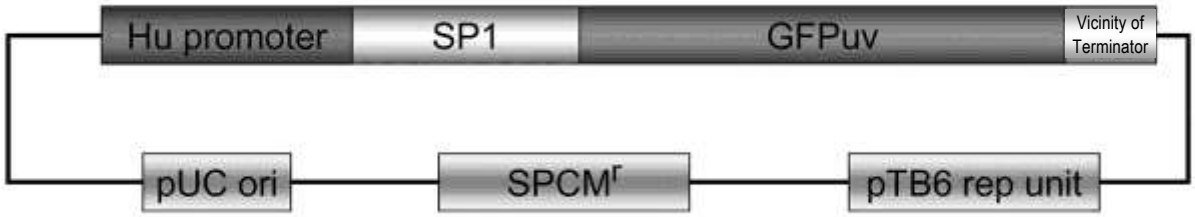
Trastuzumab-scFv-His :

```
  1 ATGGAAGTTC AGCTGGTTGA AAGCGGTGGC GGTCTGGTTC AGCCTGGTGG
 51 TAGCCTGCGT CTGAGCTGTG CAGCAAGCGG TTTTAACATT AAAGATACCT
101 ATATTCATTG GGTGCGTCAG GCACCGGGTA AAGGTCTGGA ATGGGTTGCA
151 CGTATTTATC CGACCAATGG TTATACCCGT TATGCCGATA GCGTTAAAGG
201 TCGTTTTACC ATTAGCGCAG ATACCAGCAA AAATACCGCA TATCTGCAGA
251 TGAATAGCCT GCGTGCAGAG GATACCGCAG TGTATTATTG TAGCCGTTGG
301 GGTGGTGATG GTTTTTATGC AATGGATTAT TGGGGTCAGG GCACCCTGGT
351 TACCGTTAGC AGTGGTGGTG GTGGTAGCGG TGGTGGGGGT TCAGGTGGTG
401 GTGGATCCGA TATTCAGATG ACCCAGAGCC CGAGCAGCCT GAGCGCAAGC
451 GTTGGTGATC GTGTTACCAT TACCTGTCGT GCAAGCCAGG ATGTTAATAC
501 CGCAGTTGCA TGGTATCAGC AGAAACCGGG TAAAGCACCG AAACTGCTGA
551 TTTATAGCGC AAGCTTTCTG TATAGCGGTG TTCCGAGCCG TTTTAGCGGT
601 AGCCGTAGCG GCACCGATTT TACCCTGACC ATTAGCAGCC TGCAGCCGGA
651 AGATTTTGCA ACCTATTATT GTCAGCAGCA TTACACCACC CCTCCGACCT
701 TTGGTCAGGG CACCAAAGTT GAAATTAAAC ATCATCATCA CCATCATTAA
```

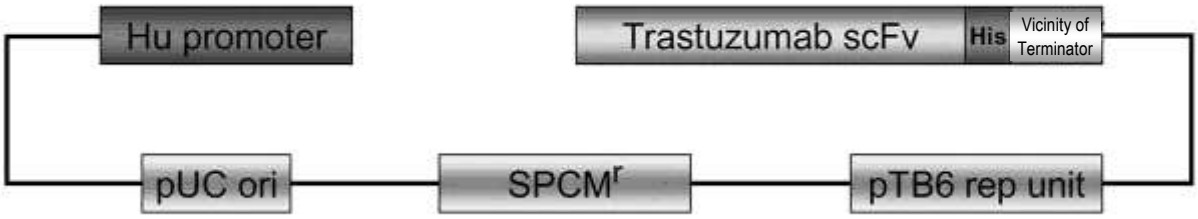
1-750 : Trastuzumab-scFv-His	SEQ ID NO: 5
1-363 : Trastuzumab-scFv VH	SEQ ID NO: 6
(292-331 : CDR3-1)	SEQ ID NO: 7
364-408 : Trastuzumab-scFv linker	SEQ ID NO: 8
409-729 : Trastuzumab-scFv VK	SEQ ID NO: 9
(673-699 : CDR3-2)	SEQ ID NO: 10
730-747 : 6xHis-tag	SEQ ID NO: 11

[Figure 3]

Plasmid SP1B-9

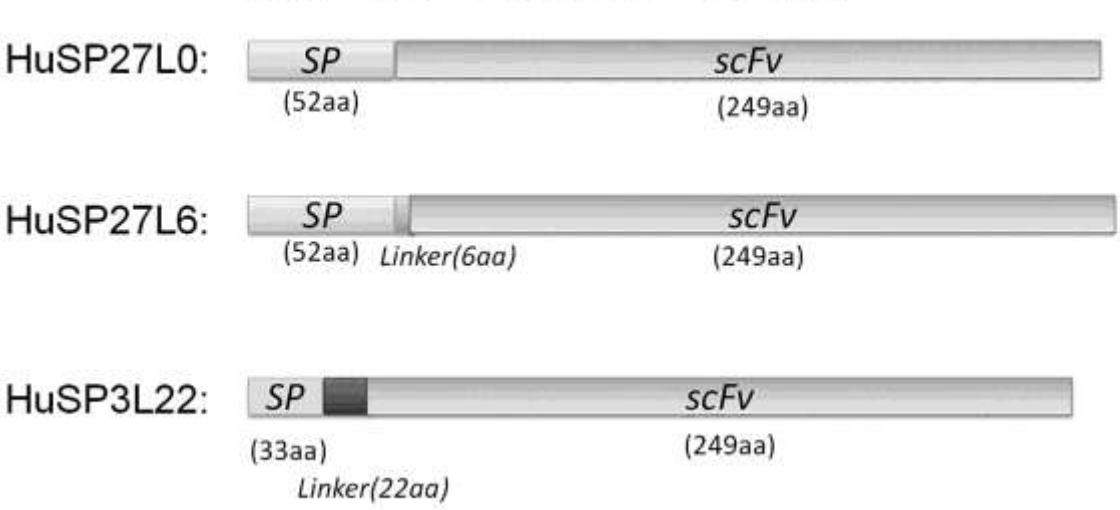


[Figure 4]



[Figure 5]

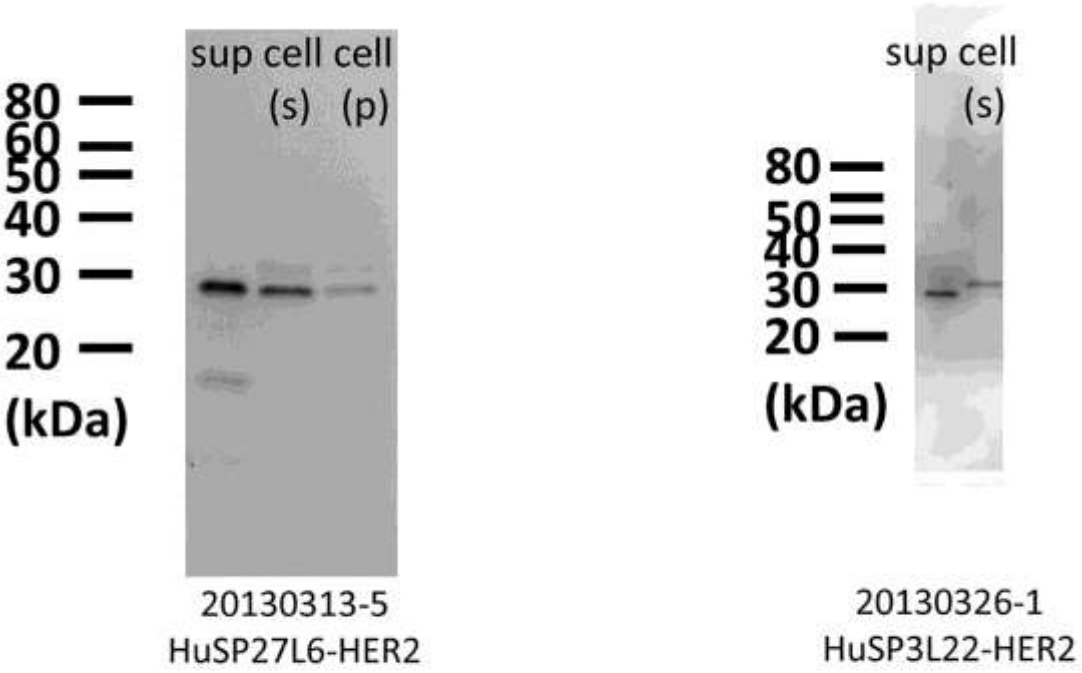
Trastuzumab scFv structure in recombinant bifidobacteria detected by Western blot analysis



Size of Trastuzumab scFv body: approximately 25 kDa

[Figure 6]

Trastuzumab scFv expression in bifidobacteria (Western blot analysis)



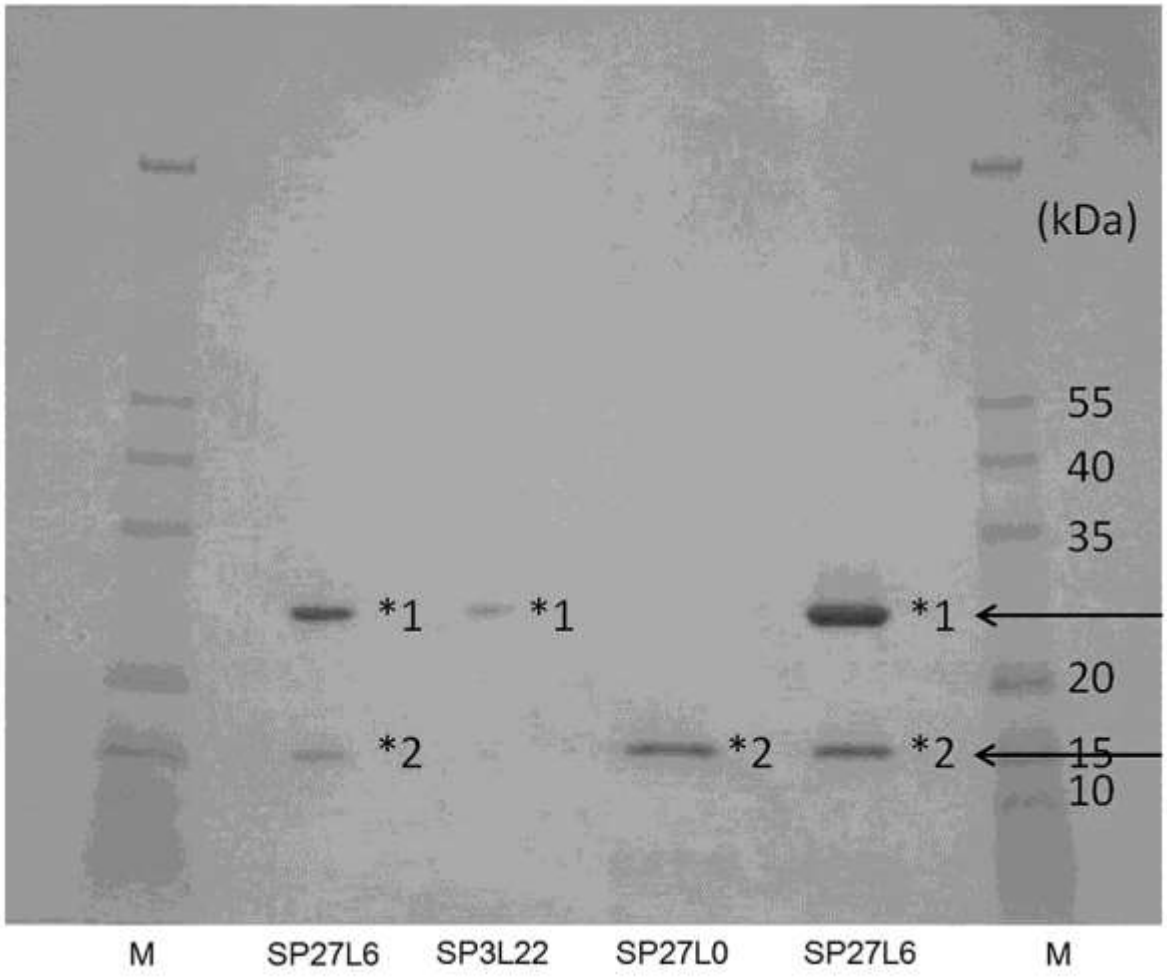
Sup: bifidobacteria culture supernatant in 500 μL of culture solution

Cell-s: bifidobacteria soluble fraction in 2.5 μL of culture solution

Cell-p: bifidobacteria insoluble fraction in 25 μL of culture solution

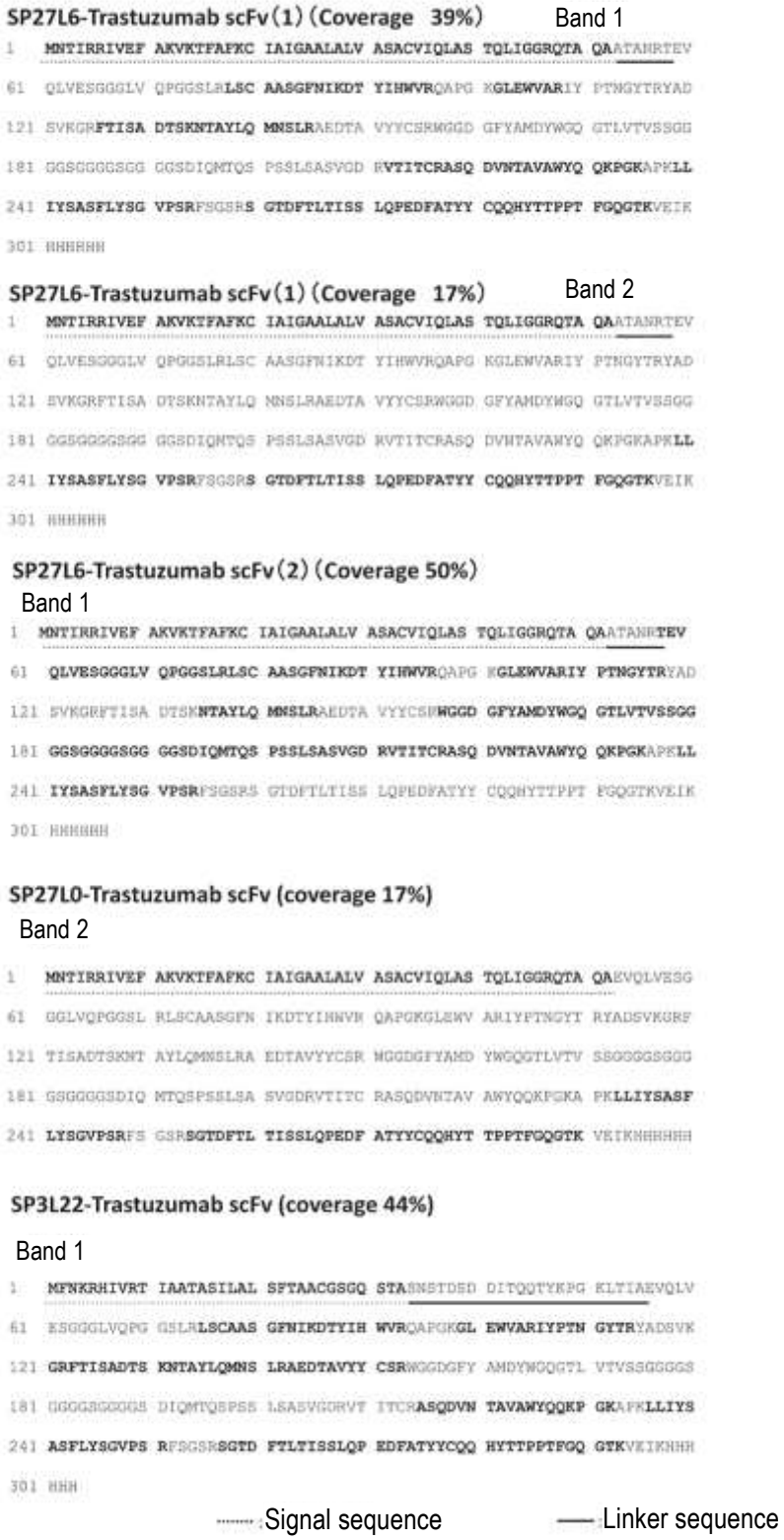
Detected using histidine tag at C-terminus as indicator

[Figure 7]



[Figure 8]

Analysis results of His-Tag-purified protein by LC-MS/MS



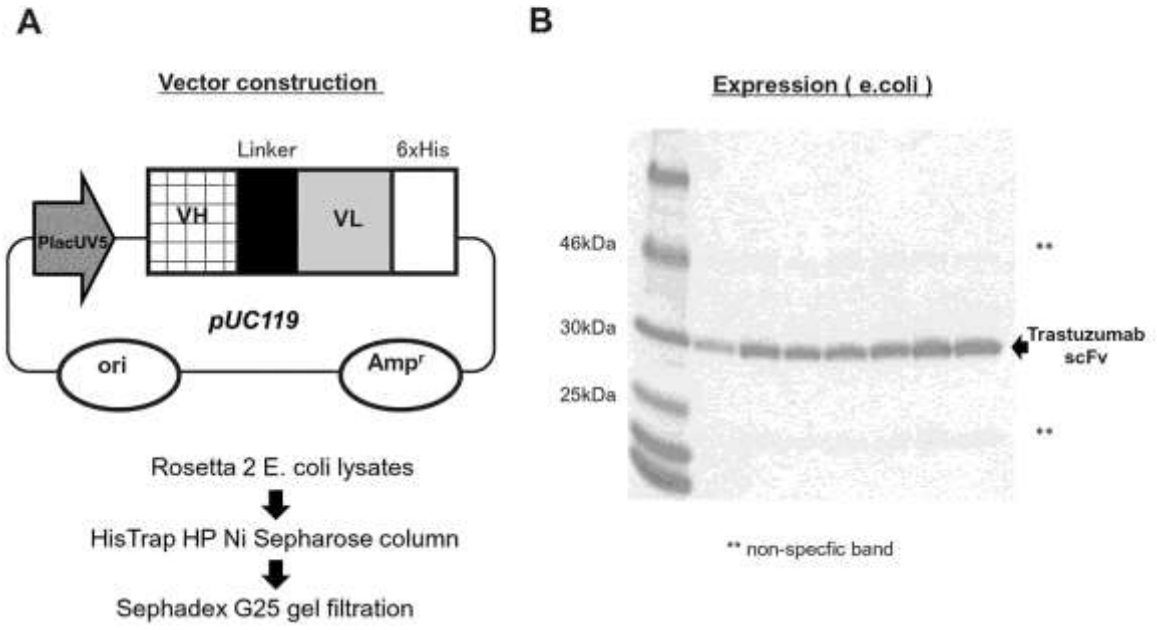
[Figure 9]

Peptide fragments matched with Trastuzumab scFv amino acid sequence by LC-MS/MS analysis

1) TEVQLVESGGGLVQPGGSLR
2) LSCAASGFNIKDTYIHWVR
3) GLEWVARIYPTNGYTR
4) NTAYLQMNSLR
5) WGGDGFYAMDYWGQGTLVTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDR
6) VTITCRASQDVNTAVAWYQQKPGK
7) LLIYSASFLYSGVPSR

[Figure 10]

Construction of Trastuzumab scFv secretion vector and expression of Trastuzumab scFv in E. coli

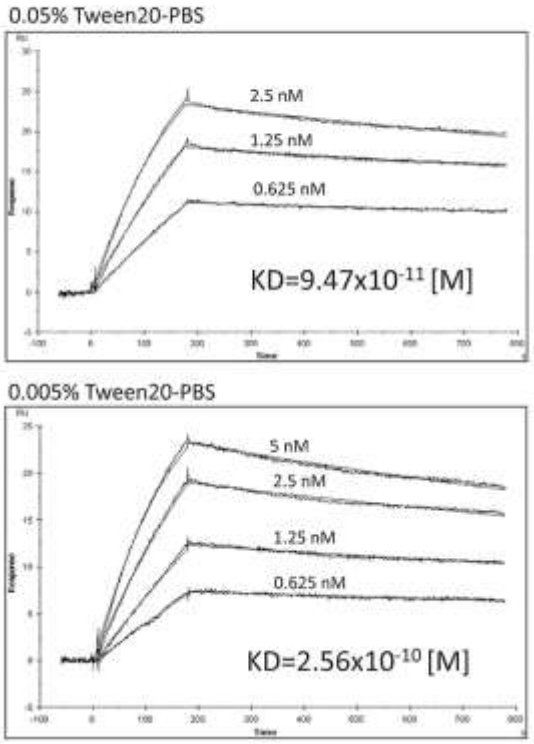


[Figure 11]

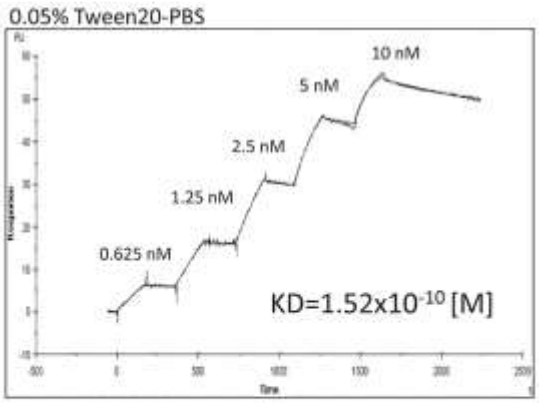
Measurement of affinity of Trastuzumab scFv with HER2 extracellular domain by Biacore X100

Multi-cycle method

Immobilized side; HER2ex.
Flow channel side; scFv



Single cycle method

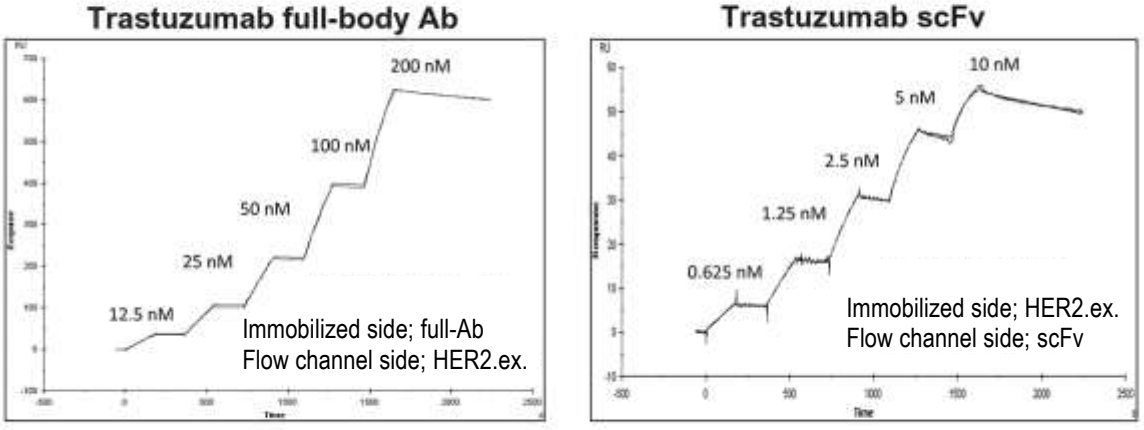


[Figure 12]

Measurement of affinity of Trastuzumab full-body antibody with HER2 extracellular domain by Biacore X100

Single cycle method

0.05% Tween20-PBS

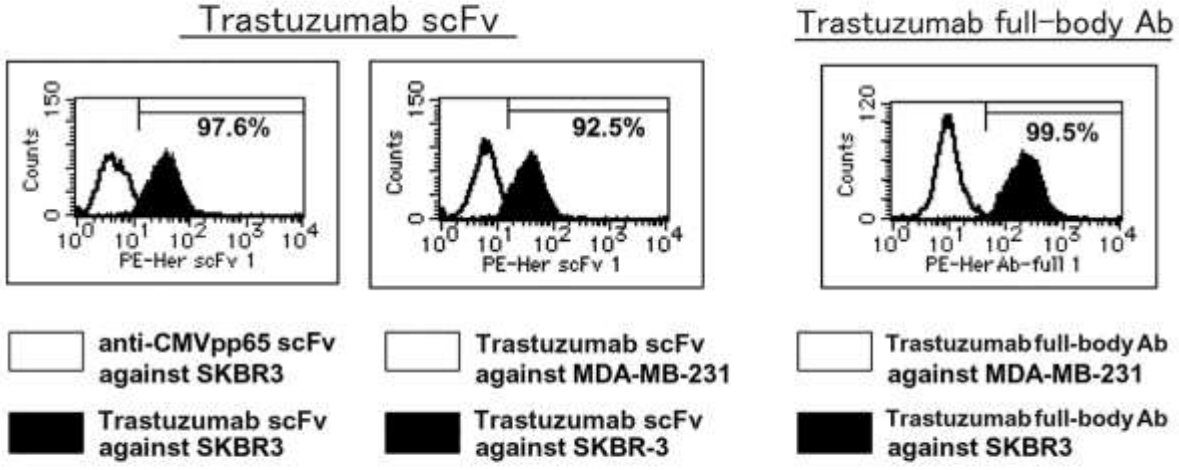


KD=3.56x10^{-9} [M]
ka=1.87x10^{4} [1/Ms]
kd=6.65x10^{-5} [1/s]

KD=1.52x10^{-10} [M]
ka=1.03x10^{6} [1/Ms]
kd=1.57x10^{-4} [1/s]

[Figure 13]

FACS analysis of binding ability of Trastuzumab scFv and Trastuzumab full-body antibody to human breast cancer cell lines

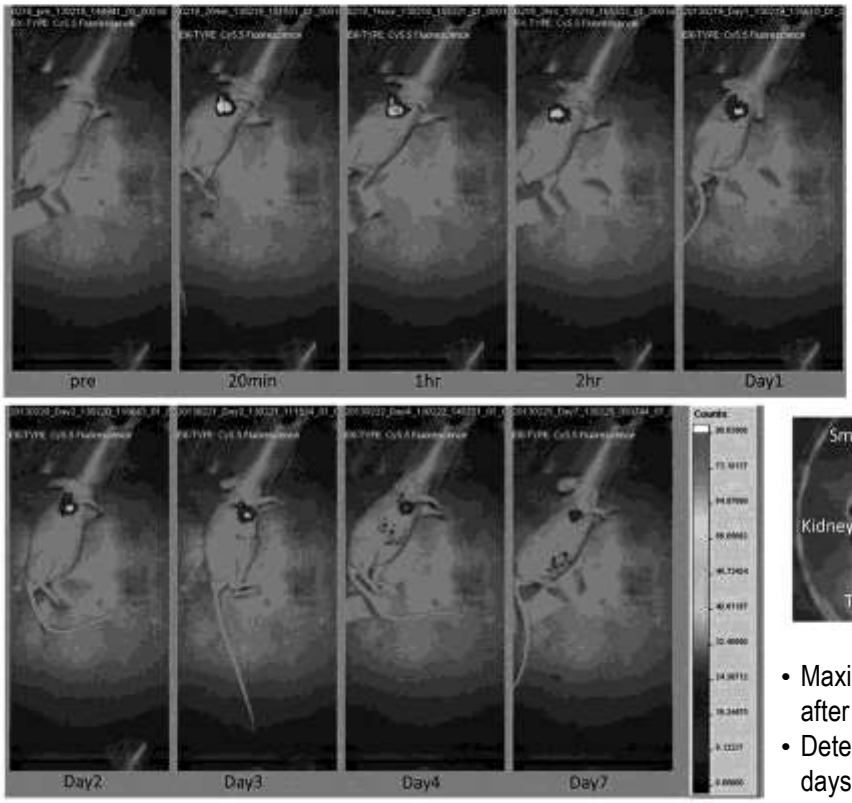


*Trastuzumab scFv and anti-CMVpp65 scFv Ab at 10ug/ml, Trastuzumab full-body Ab (neat supernatant of Sf9 cells)

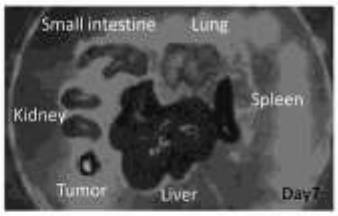
**MDA-MB-231(HER2-) SKBR-3 (HER2++)

[Figure 14]

In vivo dynamic imaging of Cy5.5-labeled Trastuzumab scFv using in vivo tumor models



- MDAMB361 $5x10^6$/animal fat pad (2013.01.08)
- Cy5.5 labelled Trastuzumab scFv intratumoral (100μL) 3.75mg/kg

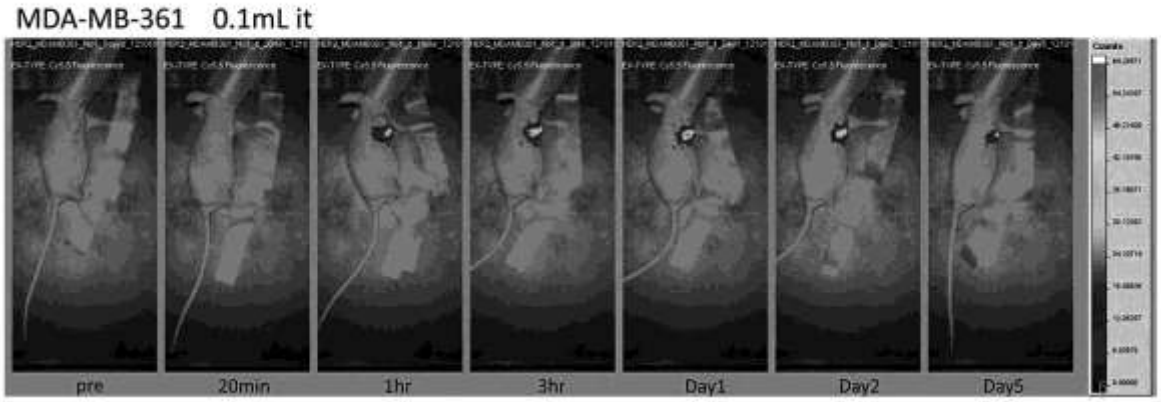


- Maximum intensity at 1 hour after administration
- Detectable even after 7 days after administration

[Figure 15]

In vivo dynamic imaging of Cy5.5-labeled Trastuzumab full-body Ab using in vivo tumor models

- Animals after transplantation of 4 x 10^6 MDA-MB-361 used
- Intratumoral administration of Cy5.5-labeled Trastuzumab full-body antibody (in amount corresponding to 3.75 mg/kg) (Day 56)
- After administration, imaging over time until 20 min, 1 hr, 3 hr, Day 1, Day 2, and Day 5

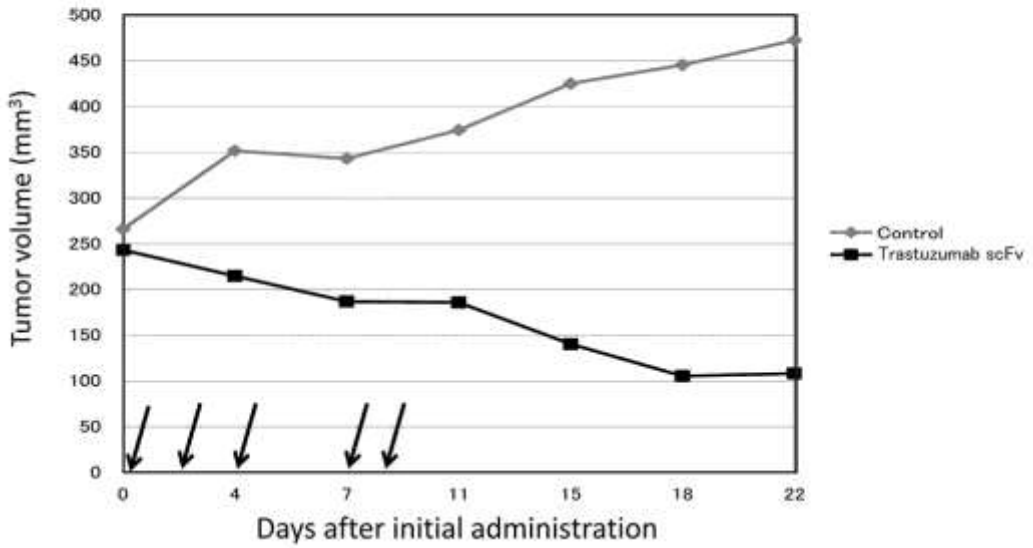


[Figure 16]

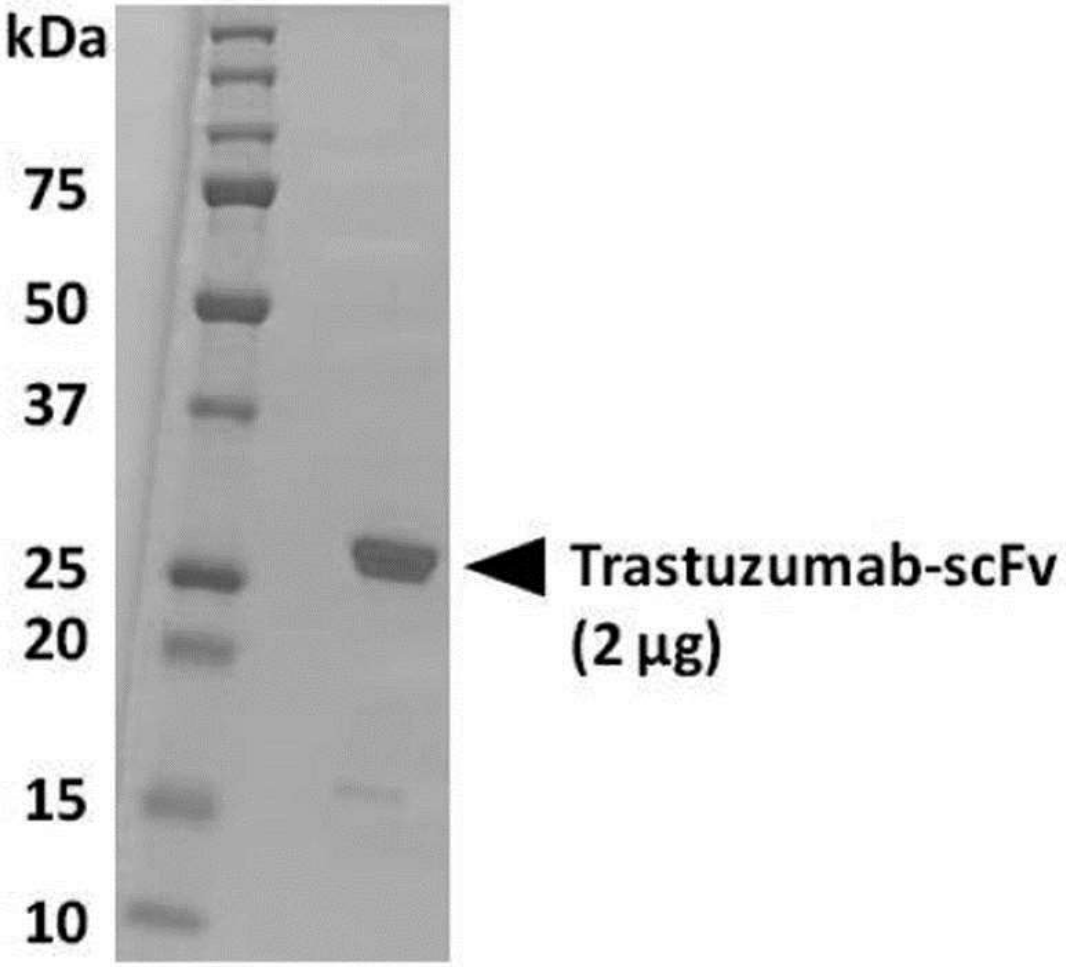
Inhibiting effect of Trastuzumab scFv on growth of orthotropic grafted tumor of human breast cancer MDA-MB-361 cells

MDA-MB-361 1x10^6 cells (contain Matrigel)/mouse, female BALB/cA-*nu/nu* mice, fad pad (n=4-5)

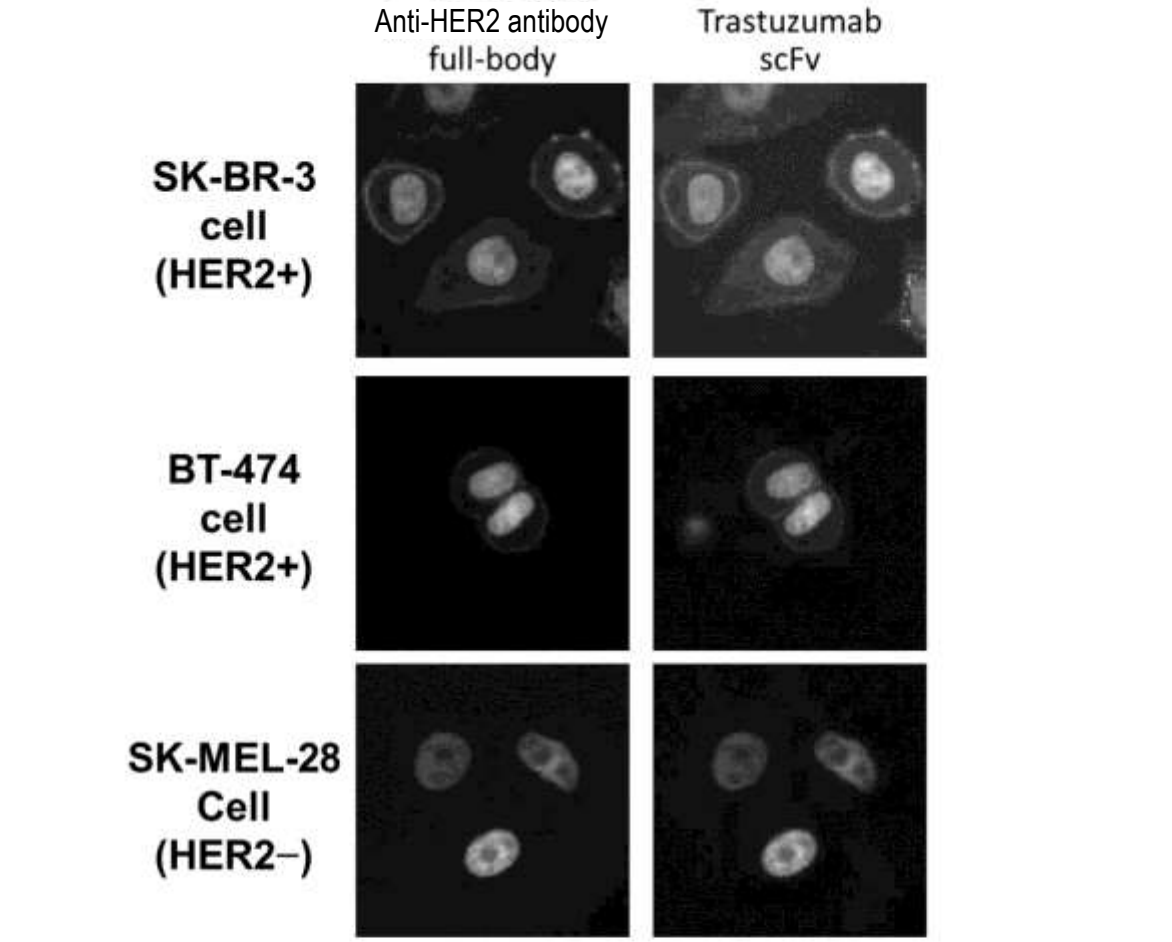
Trastuzumab scFv 3.75mg/kg (injection volume 50μL/it) x 4 (Days 0, 2, 4, 7, 9)



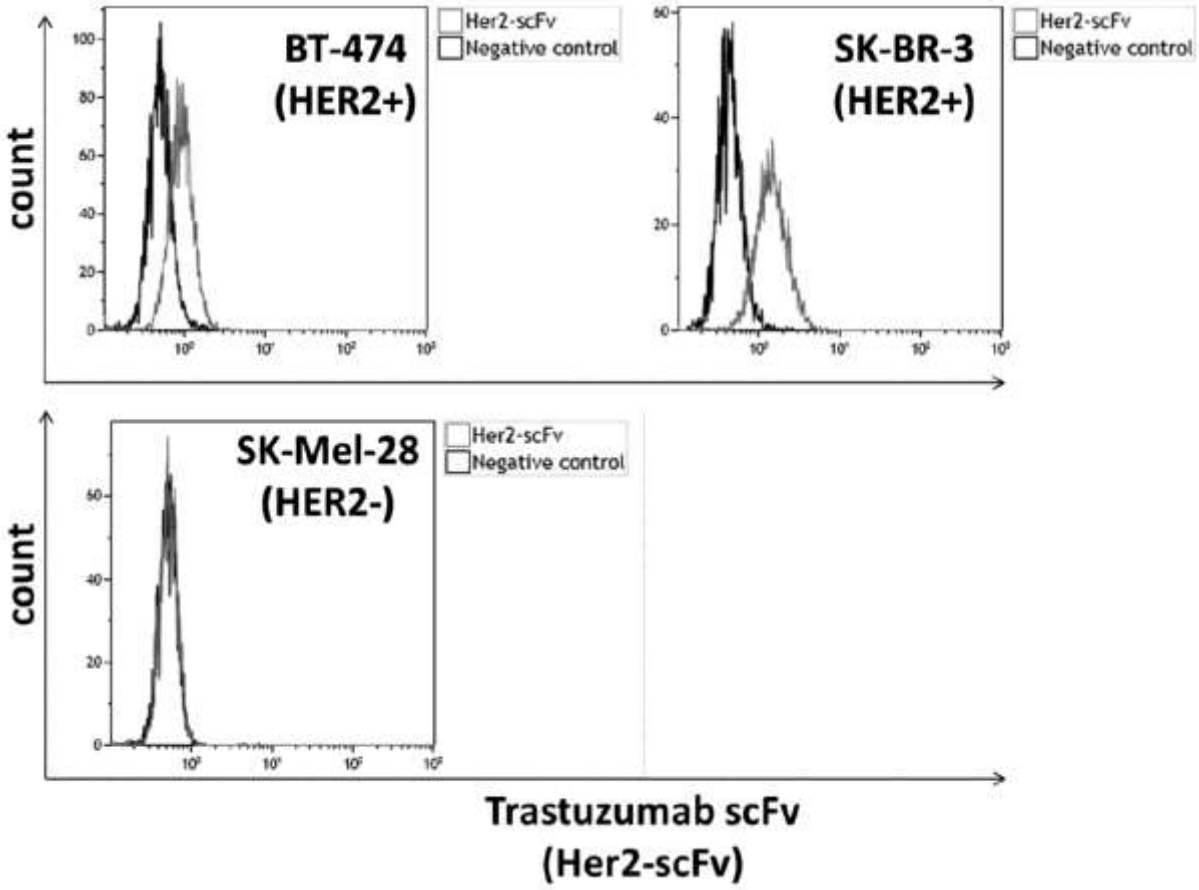
[Figure 17]



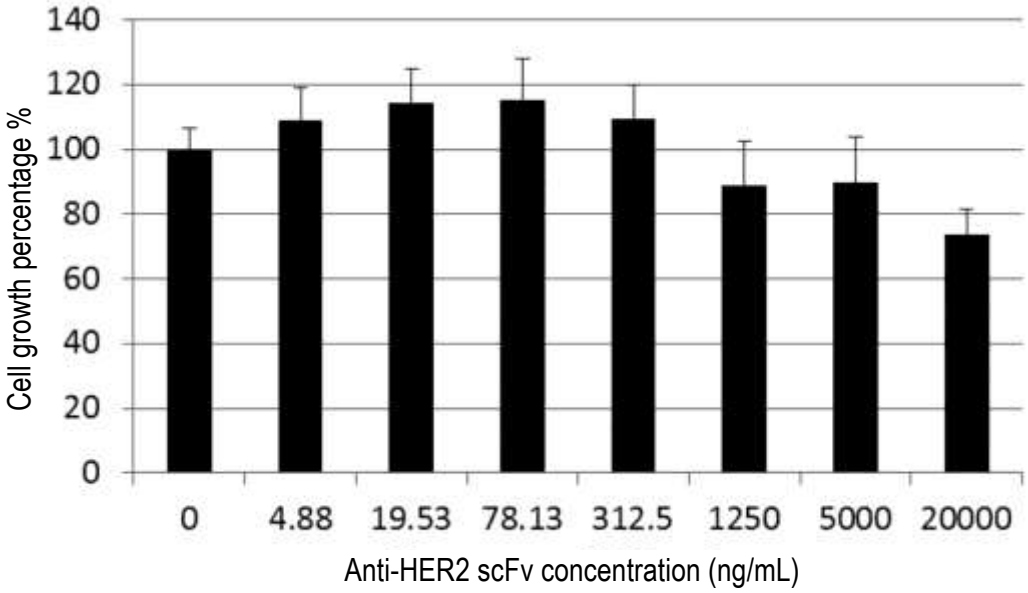
[Figure 18]


Anti-HER2 antibody full-body
Trastuzumab scFv
SK-BR-3 cell (HER2+)
BT-474 cell (HER2+)
SK-MEL-28 Cell (HER2−)
HER2 staining
Primary antibody ...Anti-HER2 antibody (full-body) (5 μg/mL) 4°C, 1h.
Secondary antibody ...DyLight 594 goat anti-mouse IgG (5 μg/mL) 4°C, 0.5h
scFv staining
Primary antibody ...B. longum Trastuzumab scFv (5 μg/mL) 4°C, 1h.
Secondary antibody ...anti-His-tag-Alexa Fluor 488 (2.5 μg/mL) 4°C, 0.5h

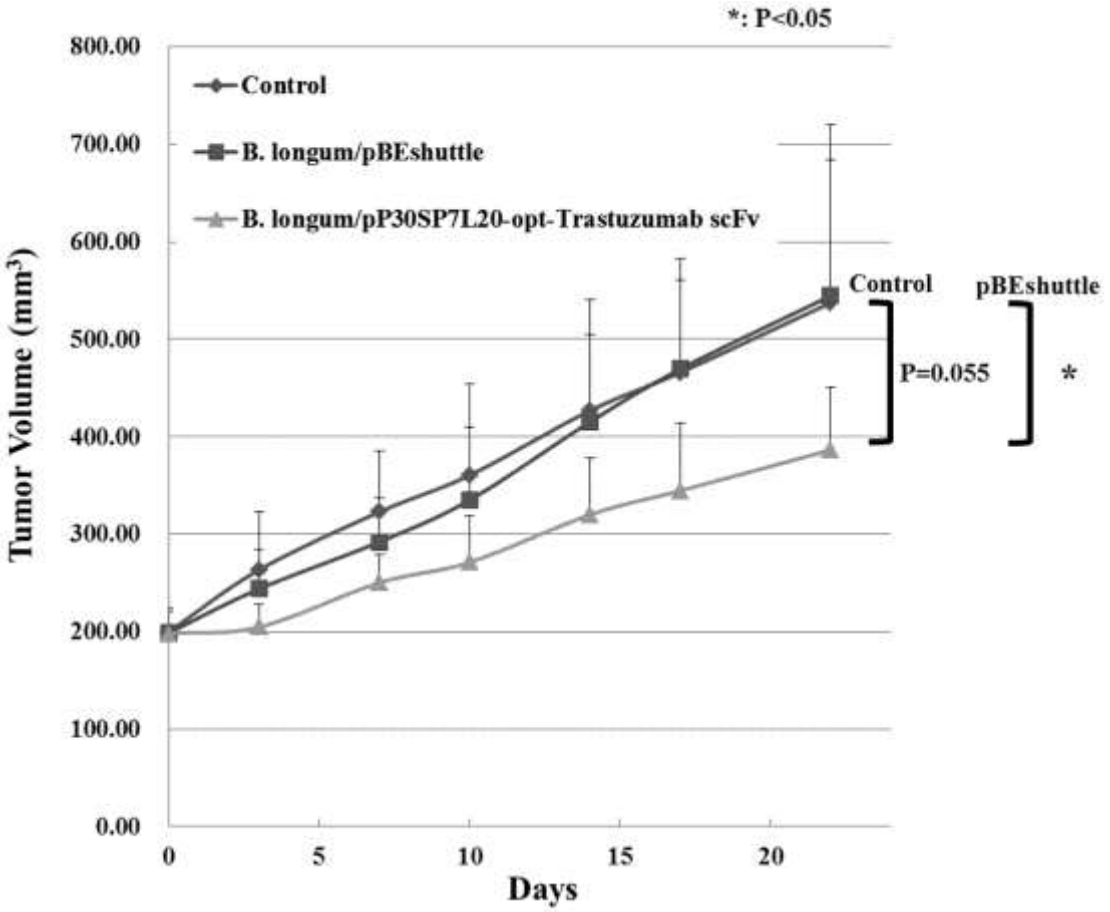
[Figure 19]



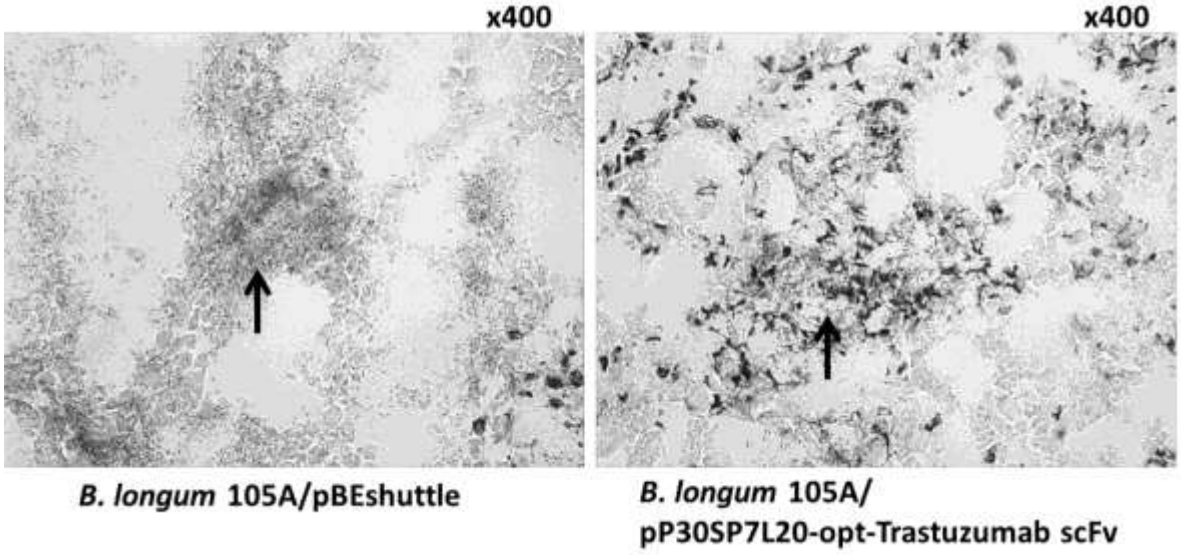
[Figure 20]



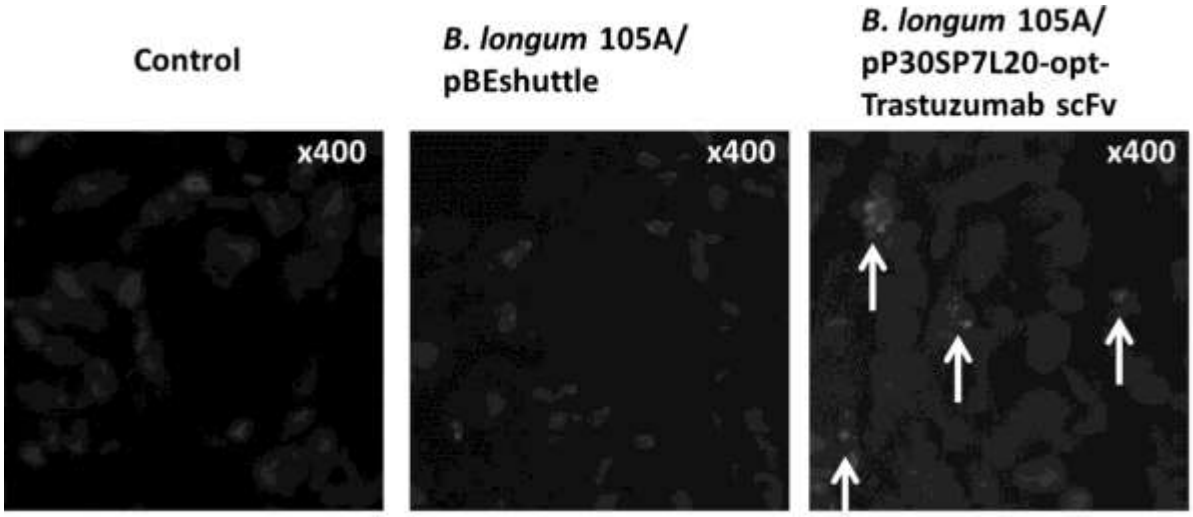
[Figure 21]



[Figure 22]



[Figure 23]


Control
B. longum 105A/ pBEshuttle
B. longum 105A/ pP30SP7L20-opt-Trastuzumab scFv
x400
x400
x400