Lab Seminar

Jan 17, 2007

Michael Jeltsch

1. What to do about the mess in our lab?

2. Contract research for Lymphatix Ltd. and Vegenics Ltd.

Adenoviral mature VEGF-D treatment

1. Comparison of different mature major VEGF-D coding sequences with respect to its influence on expression levels (optimization of codon usage, testing of a few different signal peptides)

2. Stimulation of VEGFR-2 and VEGFR-3 phosphorylation by VEGF-D with the best expressing constructs

3. Determination of the N-terminus of the mature peptide of the best expressing constructs

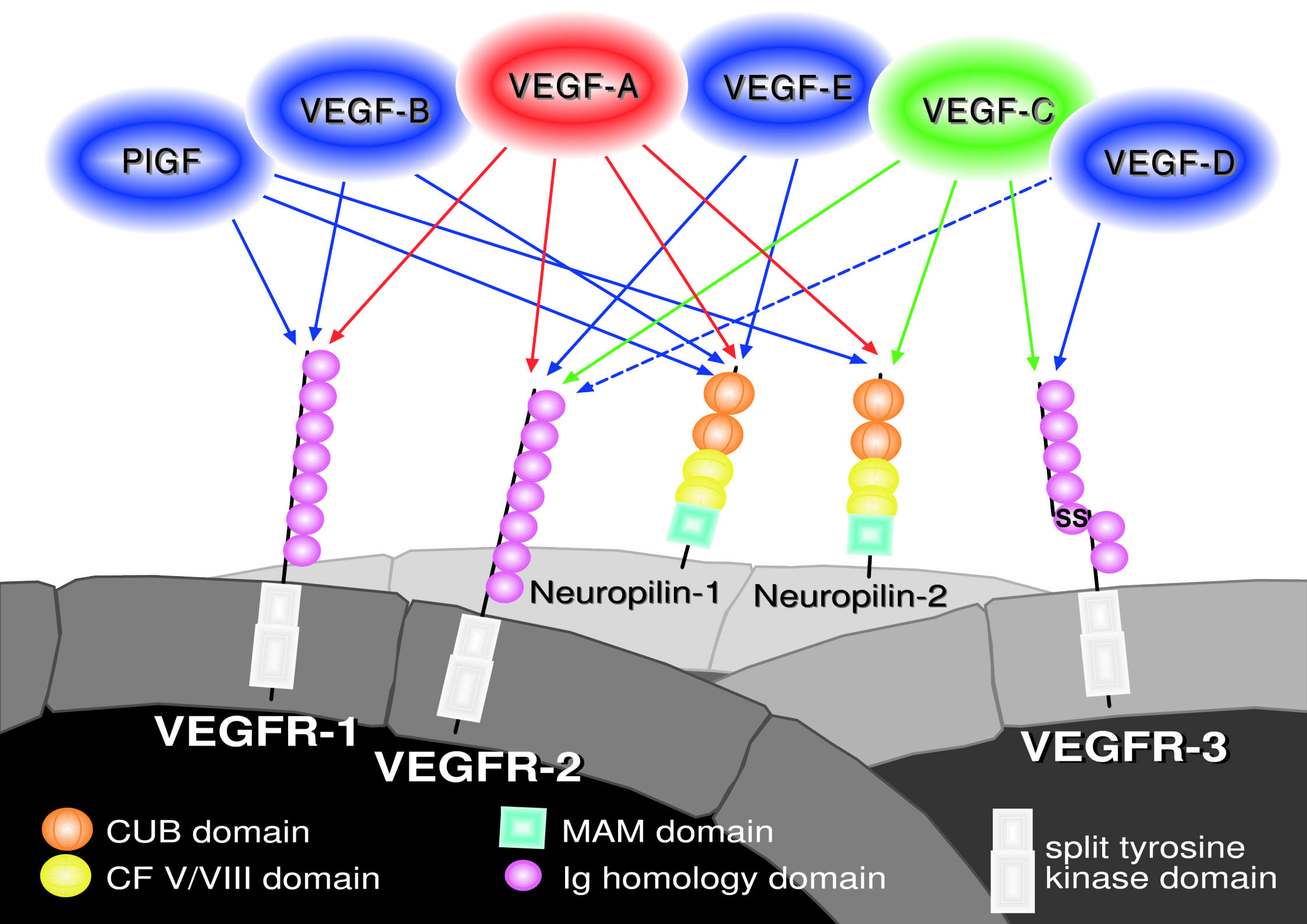
4. Removal of the histag from the highest expressing construct and creation of the adenoviral transfer vector

VEGF-D Is the Strongest Angiogenic and Lymphangiogenic Effector Among VEGFs Delivered Into Skeletal Muscle via Adenoviruses

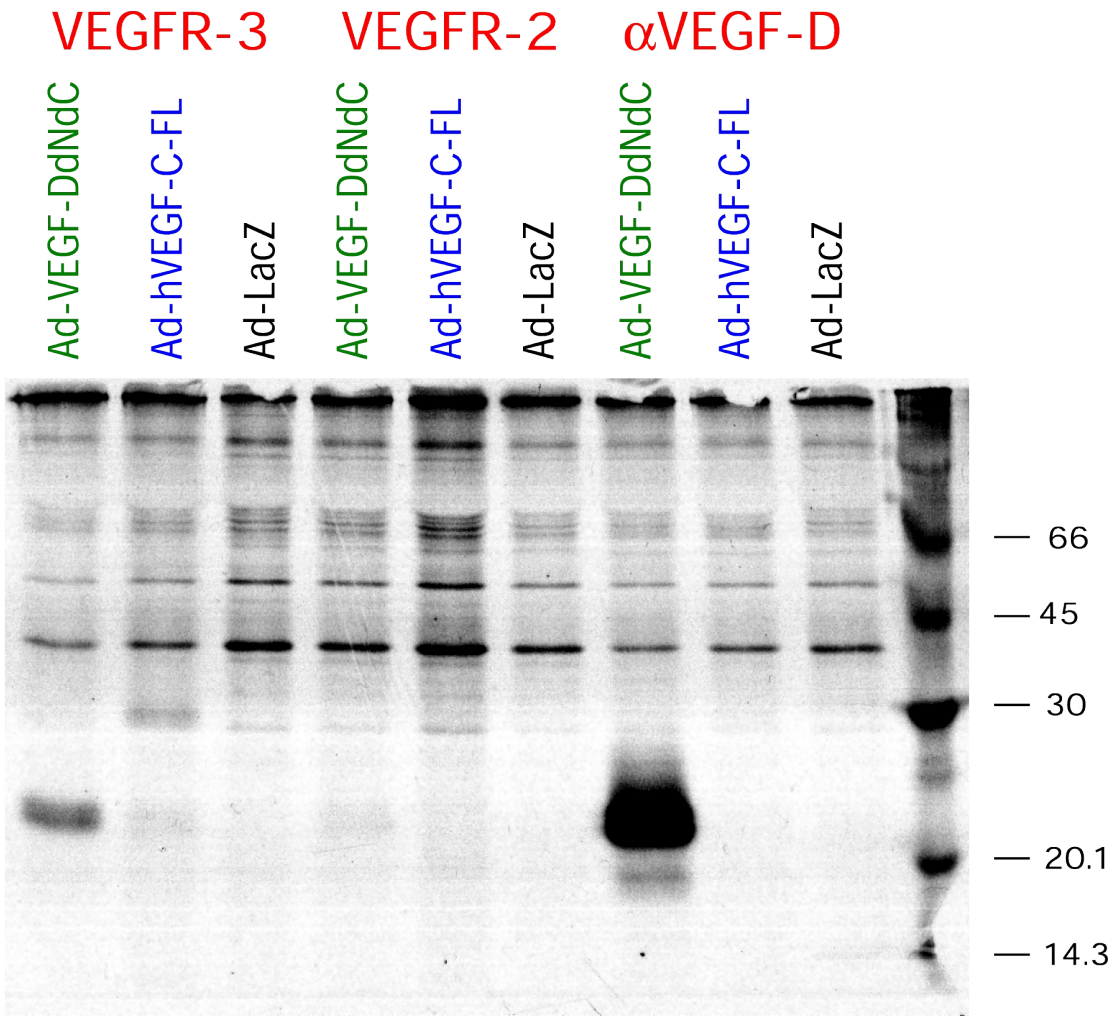
Tuomas T. Rissanen,* Johanna E. Markkanen,* Marcin Gruchala, Tommi Heikura, Antti Puranen, Mikko I. Kettunen, Ivana Kholová, Risto A. Kauppinen, Marc G. Achen, Steven A. Stacker, Kari Alitalo, Seppo Ylä-Herttuala

Abstract—Optimal angiogenic and lymphangiogenic gene therapy requires knowledge of the best growth factors for each purpose. We studied the therapeutic potential of human vascular endothelial growth factor (VEGF) family members VEGF-A, VEGF-B, VEGF-C, and VEGF-D as well as a VEGFR-3–specific mutant (VEGF-C^{156S}) using adenoviral gene transfer in rabbit hindlimb skeletal muscle. The significance of proteolytic processing of VEGF-D was explored using adenoviruses encoding either full-length or mature (ΔNΔC) VEGF-D. Adenoviruses expressing potent VEGFR-2 ligands, VEGF-A and VEGF-$D^{\Delta N\Delta C}$, induced the strongest angiogenesis and vascular permeability effects as assessed by capillary vessel and perfusion measurements, modified Miles assay, and MRI. The most significant feature of angiogenesis induced by both VEGF-A and VEGF-$D^{\Delta N\Delta C}$ was a remarkable enlargement of microvessels with efficient recruitment of pericytes suggesting formation of arterioles or venules. VEGF-A also moderately increased capillary density and created glomeruloid bodies, clusters of tortuous vessels, whereas VEGF-$D^{\Delta N\Delta C}$–induced angiogenesis was more diffuse. Vascular smooth muscle cell proliferation occurred in regions with increased plasma protein extravasation, indicating that arteriogenesis may be promoted by VEGF-A and VEGF-$D^{\Delta N\Delta C}$. Full-length VEGF-C and VEGF-D induced predominantly and the selective VEGFR-3 ligand VEGF-C^{156S} exclusively lymphangiogenesis. Unlike angiogenesis, lymphangiogenesis was not dependent on nitric oxide. The VEGFR-1 ligand VEGF-B did not promote either angiogenesis or lymphangiogenesis. Finally, we found a positive correlation between capillary size and vascular permeability. This study compares, for the first time, angiogenesis and lymphangiogenesis induced by gene transfer of different human VEGFs, and shows that VEGF-D is the most potent member when delivered via an adenoviral vector into skeletal muscle. (***Circ Res*. 2003;92:1098-1106.**)

Key Words: vascular permeability ■ magnetic resonance imaging ■ edema ■ pericyte ■ arteriogenesis


PlGF
VEGF-B
VEGF-A
VEGF-E
VEGF-C
VEGF-D
Neuropilin-1
Neuropilin-2
VEGFR-1
VEGFR-2
VEGFR-3
CUB domain
CF V/VIII domain
MAM domain
Ig homology domain
split tyrosine kinase domain

Ad-VEGF-DdNdC expression in HeLa cells and VEGFR-2/3 binding

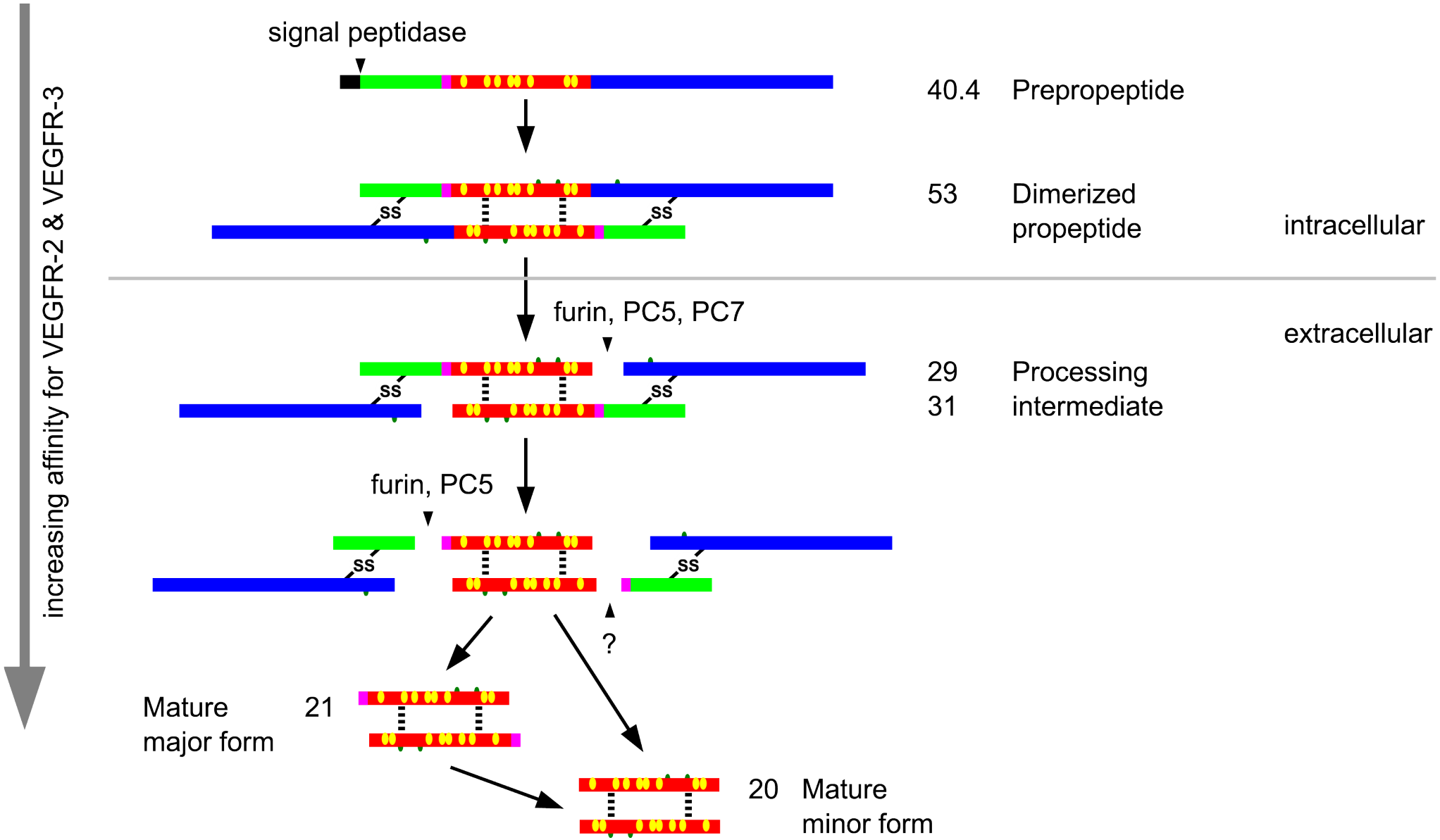


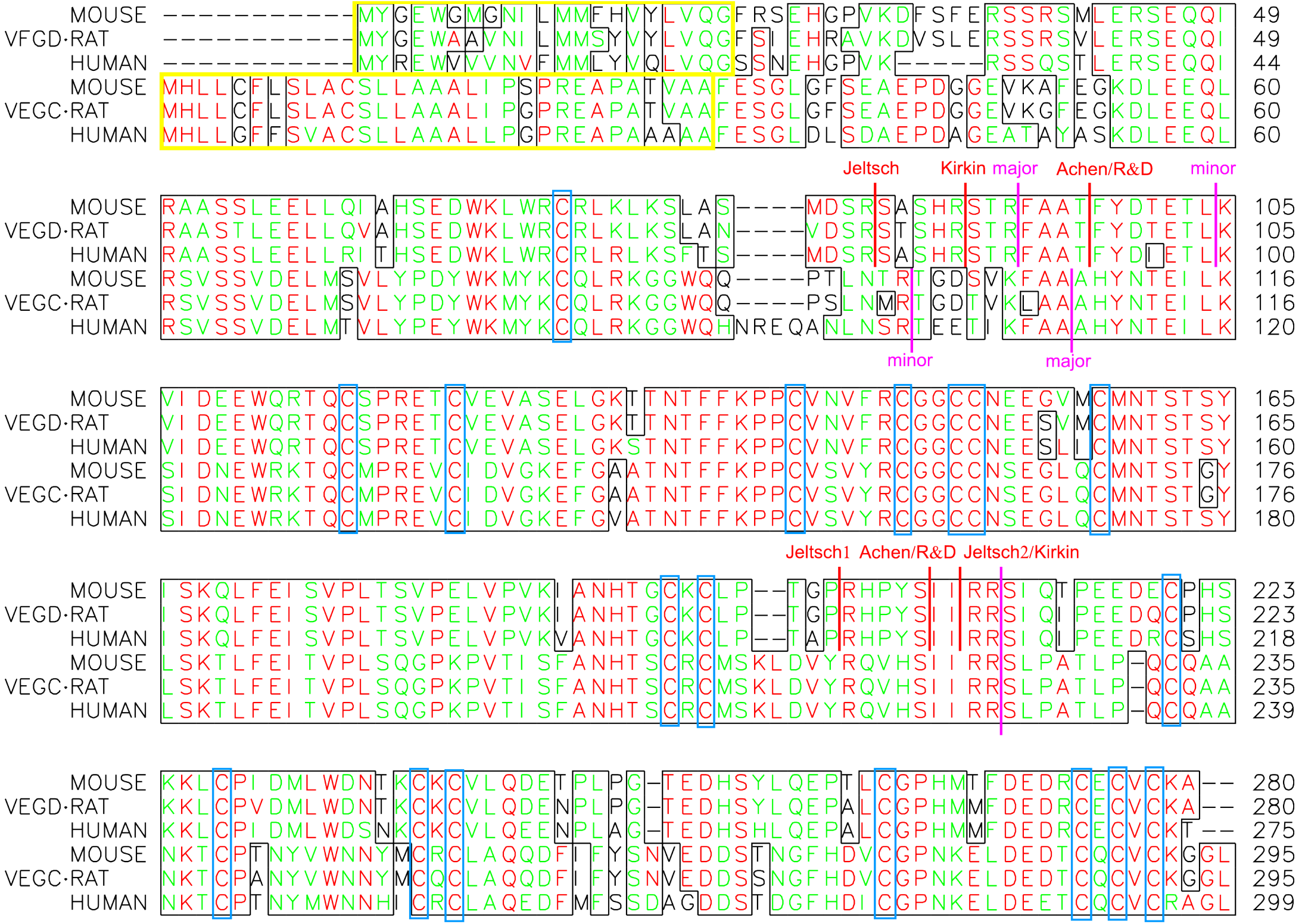
AdLacZ

Ad-VEGF-C-FL
Lot 256
2.3×10^{10} PFU/ml

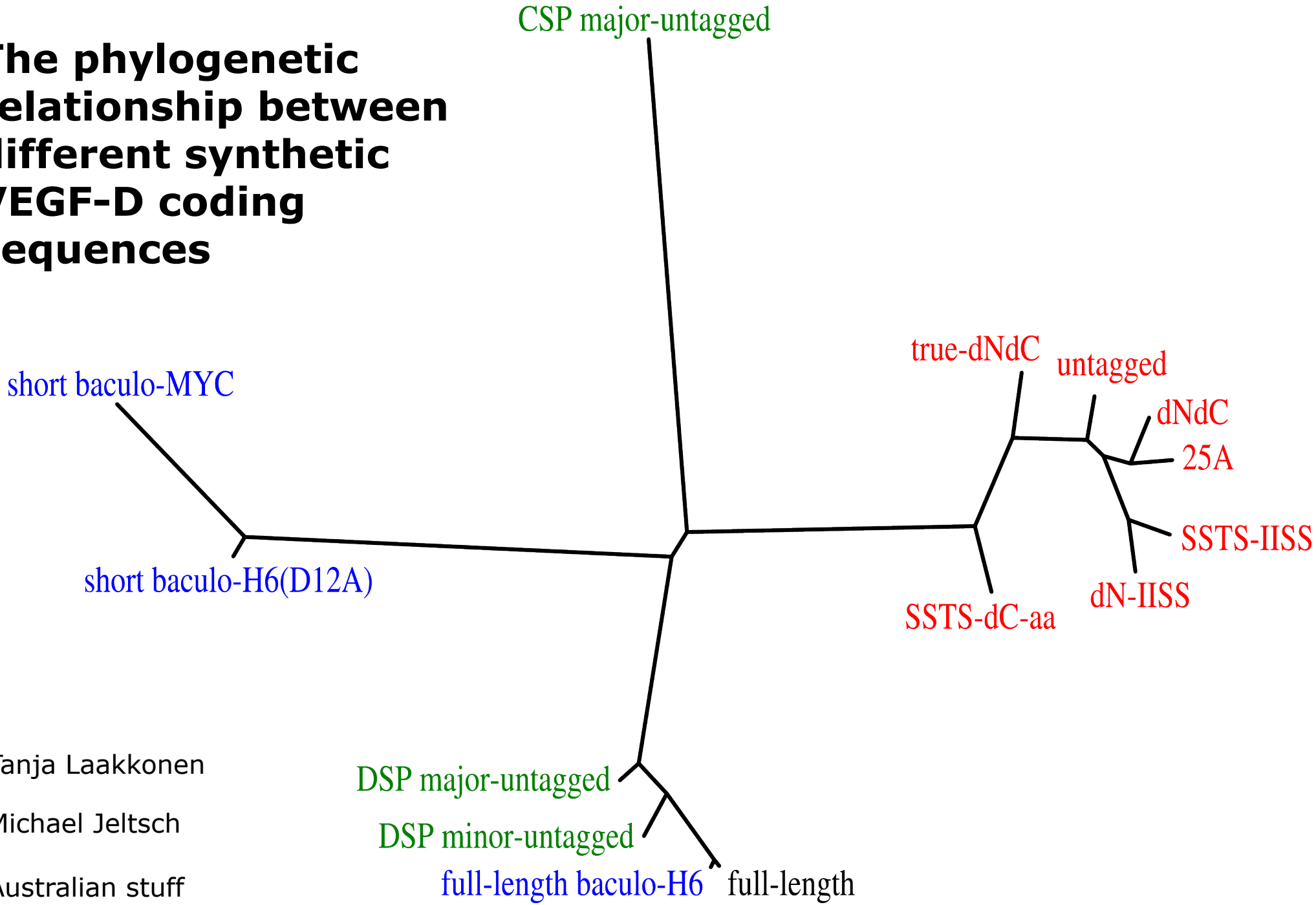
Ad-VEGF-DdNdC
Lot 375
3.0×10^{10} PFU/ml

MOI 200


increasing affinity for VEGFR-2 & VEGFR-3
signal peptidase
40.4
Prepropeptide
ss
ss
53
Dimerized
propeptide
intracellular
extracellular
furin, PC5, PC7
ss
ss
29
31
Processing
intermediate
furin, PC5
ss
ss
?
Mature
major form
21
20
Mature
minor form


MOUSE
VFGD·RAT
HUMAN
MOUSE
VEGC·RAT
HUMAN
Jeltsch
Kirkin major
Achen/R&D
minor
MOUSE
VEGD·RAT
HUMAN
MOUSE
VEGC·RAT
HUMAN
minor
major
MOUSE
VEGD·RAT
HUMAN
MOUSE
VEGC·RAT
HUMAN
Jeltsch1
Achen/R&D
Jeltsch2/Kirkin
MOUSE
VEGD·RAT
HUMAN
MOUSE
VEGC·RAT
HUMAN
MOUSE
VEGD·RAT
HUMAN
MOUSE
VEGC·RAT
HUMAN

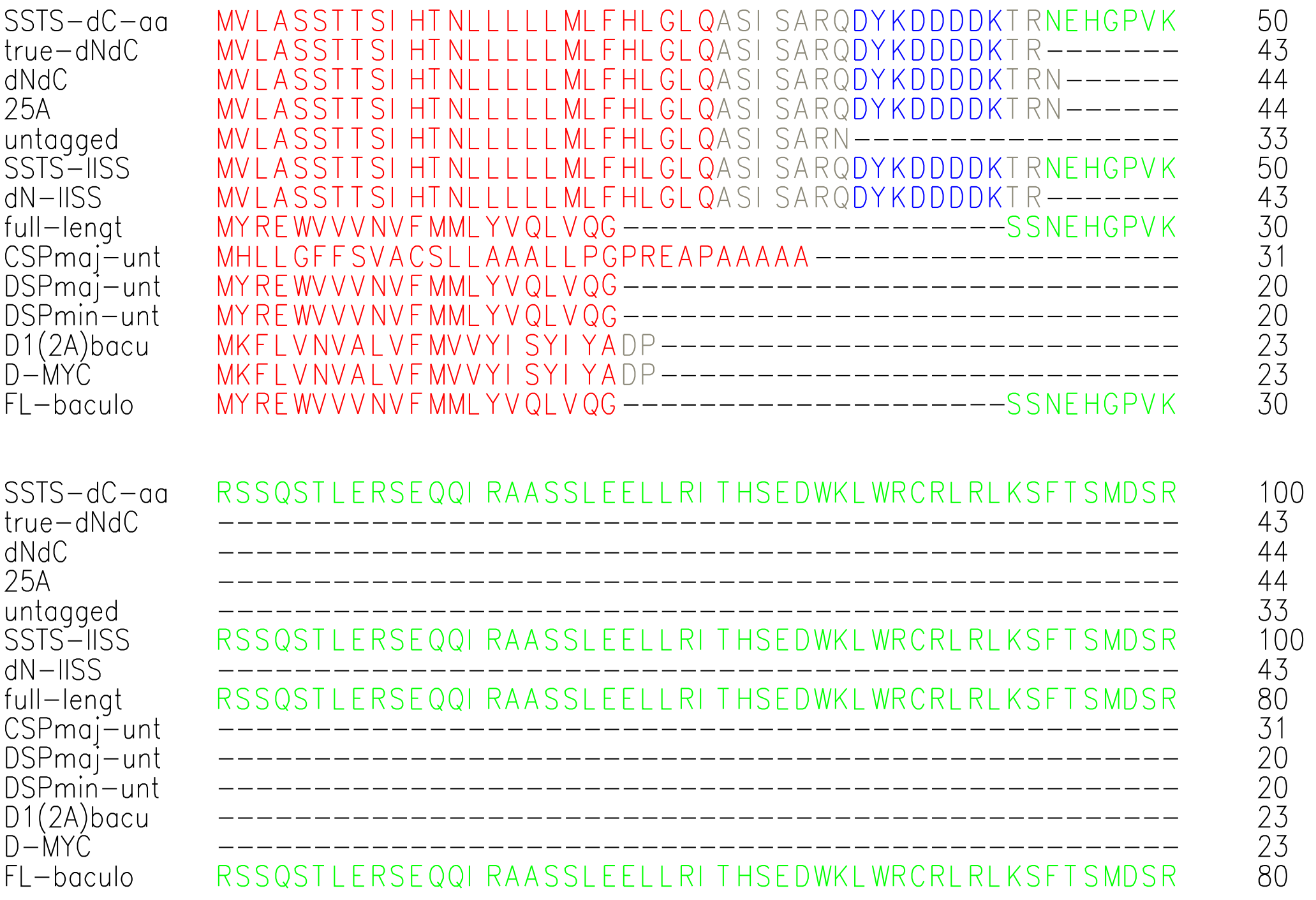
The phylogenetic relationship between different synthetic VEGF-D coding sequences

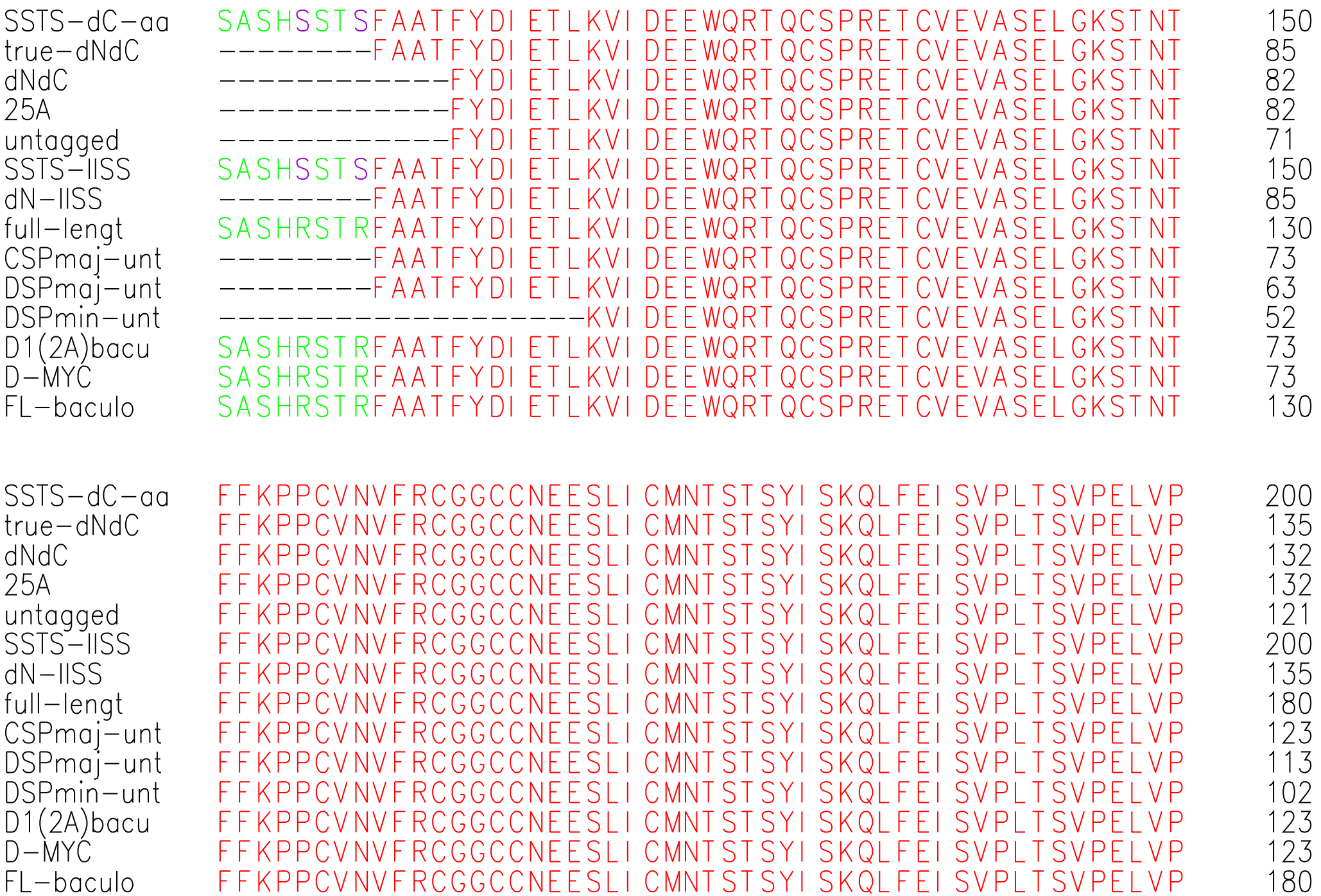


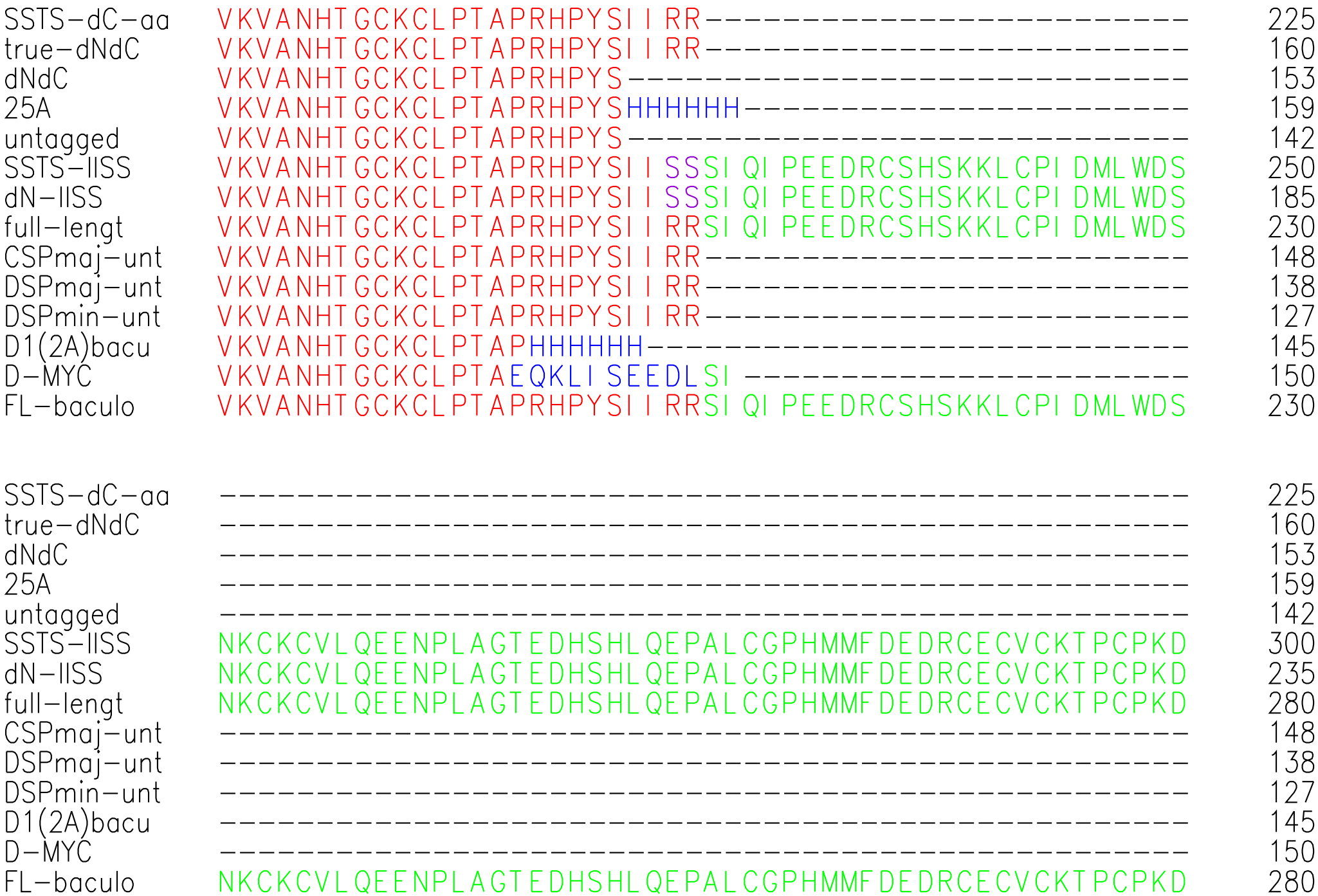
Tanja Laakkonen

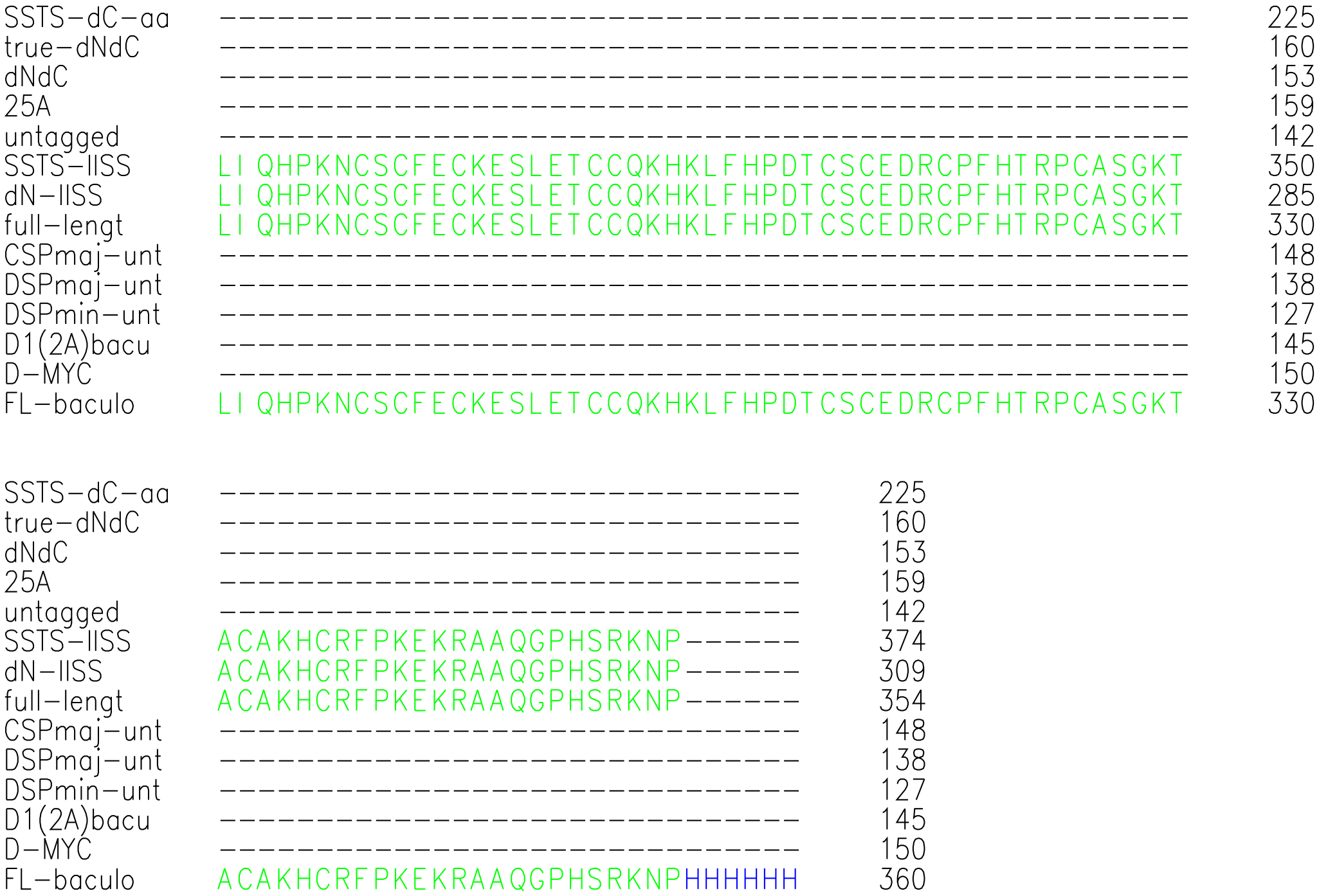
Michael Jeltsch

Australian stuff


SSTS-dC-aa MVLASSTTSIHTNLLLLLMLFHLGLQASISARQDYKDDDDKTRNEHGPVK 50
true-dNdC MVLASSTTSIHTNLLLLLMLFHLGLQASISARQDYKDDDDKTR------- 43
dNdC MVLASSTTSIHTNLLLLLMLFHLGLQASISARQDYKDDDDKTRN------ 44
25A MVLASSTTSIHTNLLLLLMLFHLGLQASISARQDYKDDDDKTRN------ 44
untagged MVLASSTTSIHTNLLLLLMLFHLGLQASISARN---------- 33
SSTS-IISS MVLASSTTSIHTNLLLLLMLFHLGLQASISARQDYKDDDDKTRNEHGPVK 50
dN-IISS MVLASSTTSIHTNLLLLLMLFHLGLQASISARQDYKDDDDKTR------- 43
full-lengt MYREWVVVNVFMMLYVQLVQG----------SSNEHGPVK 30
CSPmaj-unt MHLLGFFSVACSLLAAALLPGPREAPAAAAA---------- 31
DSPmaj-unt MYREWVVVNVFMMLYVQLVQG---------- 20
DSPmin-unt MYREWVVVNVFMMLYVQLVQG---------- 20
D1(2A)bacu MKFLVNVALVFMVVYISYIYADP---------- 23
D-MYC MKFLVNVALVFMVVYISYIYADP---------- 23
FL-baculo MYREWVVVNVFMMLYVQLVQG----------SSNEHGPVK 30
SSTS-dC-aa RSSQSTLERSEQQIRAASSLEELLRITHSEDWKLWRCRLRLKSFTSMDSR 100
true-dNdC ---------- 43
dNdC ---------- 44
25A ---------- 44
untagged ---------- 33
SSTS-IISS RSSQSTLERSEQQIRAASSLEELLRITHSEDWKLWRCRLRLKSFTSMDSR 100
dN-IISS ---------- 43
full-lengt RSSQSTLERSEQQIRAASSLEELLRITHSEDWKLWRCRLRLKSFTSMDSR 80
CSPmaj-unt ---------- 31
DSPmaj-unt ---------- 20
DSPmin-unt ---------- 20
D1(2A)bacu ---------- 23
D-MYC ---------- 23
FL-baculo RSSQSTLERSEQQIRAASSLEELLRITHSEDWKLWRCRLRLKSFTSMDSR 80


SSTS-dC-aa SASHSSTSFAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 150
true-dNdC ---------FAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 85
dNdC ----------FYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 82
25A ----------FYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 82
untagged ----------FYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 71
SSTS-IISS SASHSSTSFAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 150
dN-IISS ---------FAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 85
full-lengt SASHRSTRFAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 130
CSPmaj-unt ---------FAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 73
DSPmaj-unt ---------FAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 63
DSPmin-unt --------------------KVIDEEWQRTQCSPRETCVEVASELGKSTNT 52
D1(2A)bacu SASHRSTRFAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 73
D-MYC SASHRSTRFAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 73
FL-baculo SASHRSTRFAATFYDIETLKVIDEEWQRTQCSPRETCVEVASELGKSTNT 130
SSTS-dC-aa FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 200
true-dNdC FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 135
dNdC FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 132
25A FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 132
untagged FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 121
SSTS-IISS FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 200
dN-IISS FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 135
full-lengt FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 180
CSPmaj-unt FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 123
DSPmaj-unt FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 113
DSPmin-unt FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 102
D1(2A)bacu FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 123
D-MYC FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 123
FL-baculo FFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVPLTSVPELVP 180


SSTS-dC-aa VKVANHTGCKCLPTAPRHPYSIIRR---------- 225
true-dNdC VKVANHTGCKCLPTAPRHPYSIIRR---------- 160
dNdC VKVANHTGCKCLPTAPRHPYS---------- 153
25A VKVANHTGCKCLPTAPRHPYSHHHHHH---------- 159
untagged VKVANHTGCKCLPTAPRHPYS---------- 142
SSTS-IISS VKVANHTGCKCLPTAPRHPYSIISSSIQIPEEDRCSHSKKLCPIDMLWDS 250
dN-IISS VKVANHTGCKCLPTAPRHPYSIISSSIQIPEEDRCSHSKKLCPIDMLWDS 185
full-lengt VKVANHTGCKCLPTAPRHPYSIIRRSIQIPEEDRCSHSKKLCPIDMLWDS 230
CSPmaj-unt VKVANHTGCKCLPTAPRHPYSIIRR---------- 148
DSPmaj-unt VKVANHTGCKCLPTAPRHPYSIIRR---------- 138
DSPmin-unt VKVANHTGCKCLPTAPRHPYSIIRR---------- 127
D1(2A)bacu VKVANHTGCKCLPTAPHHHHHH---------- 145
D-MYC VKVANHTGCKCLPTAEQKLISEEDLSI---------- 150
FL-baculo VKVANHTGCKCLPTAPRHPYSIIRRSIQIPEEDRCSHSKKLCPIDMLWDS 230
SSTS-dC-aa ---------- 225
true-dNdC ---------- 160
dNdC ---------- 153
25A ---------- 159
untagged ---------- 142
SSTS-IISS NKCKCVLQEENPLAGTEDHSHLQEPALCGPHMMFDEDRCECVCKTPCPKD 300
dN-IISS NKCKCVLQEENPLAGTEDHSHLQEPALCGPHMMFDEDRCECVCKTPCPKD 235
full-lengt NKCKCVLQEENPLAGTEDHSHLQEPALCGPHMMFDEDRCECVCKTPCPKD 280
CSPmaj-unt ---------- 148
DSPmaj-unt ---------- 138
DSPmin-unt ---------- 127
D1(2A)bacu ---------- 145
D-MYC ---------- 150
FL-baculo NKCKCVLQEENPLAGTEDHSHLQEPALCGPHMMFDEDRCECVCKTPCPKD 280


SSTS-dC-aa ---------- 225
true-dNdC ---------- 160
dNdC ---------- 153
25A ---------- 159
untagged ---------- 142
SSTS-IISS LIQHPKNCSCFECKESLETCCQKHKLFHPDTCSCEDRCPFHTRPCASGKT 350
dN-IISS LIQHPKNCSCFECKESLETCCQKHKLFHPDTCSCEDRCPFHTRPCASGKT 285
full-lengt LIQHPKNCSCFECKESLETCCQKHKLFHPDTCSCEDRCPFHTRPCASGKT 330
CSPmaj-unt ---------- 148
DSPmaj-unt ---------- 138
DSPmin-unt ---------- 127
D1(2A)bacu ---------- 145
D-MYC ---------- 150
FL-baculo LIQHPKNCSCFECKESLETCCQKHKLFHPDTCSCEDRCPFHTRPCASGKT 330
SSTS-dC-aa ---------- 225
true-dNdC ---------- 160
dNdC ---------- 153
25A ---------- 159
untagged ---------- 142
SSTS-IISS ACAKHCRFPKEKRAAQGPHSRKNP------ 374
dN-IISS ACAKHCRFPKEKRAAQGPHSRKNP------ 309
full-lengt ACAKHCRFPKEKRAAQGPHSRKNP------ 354
CSPmaj-unt ---------- 148
DSPmaj-unt ---------- 138
DSPmin-unt ---------- 127
D1(2A)bacu ---------- 145
D-MYC ---------- 150
FL-baculo ACAKHCRFPKEKRAAQGPHSRKNPHHHHHH 360

Pitfalls:

1. How do we detect & quantitate VEGF-D, which has a very low expression level?

- No well characterized antibodies against VEGF-D
- VEGF receptors cannot be used either
- A tag is the only possibility
- A tag is not failsafe (a tag might get cleaved off or influence expression levels)

2. What do we compare against?
The corresponding forms of VEGF-C, which have never been expressed.

3. All constructs should be cloned into the same expression vector.

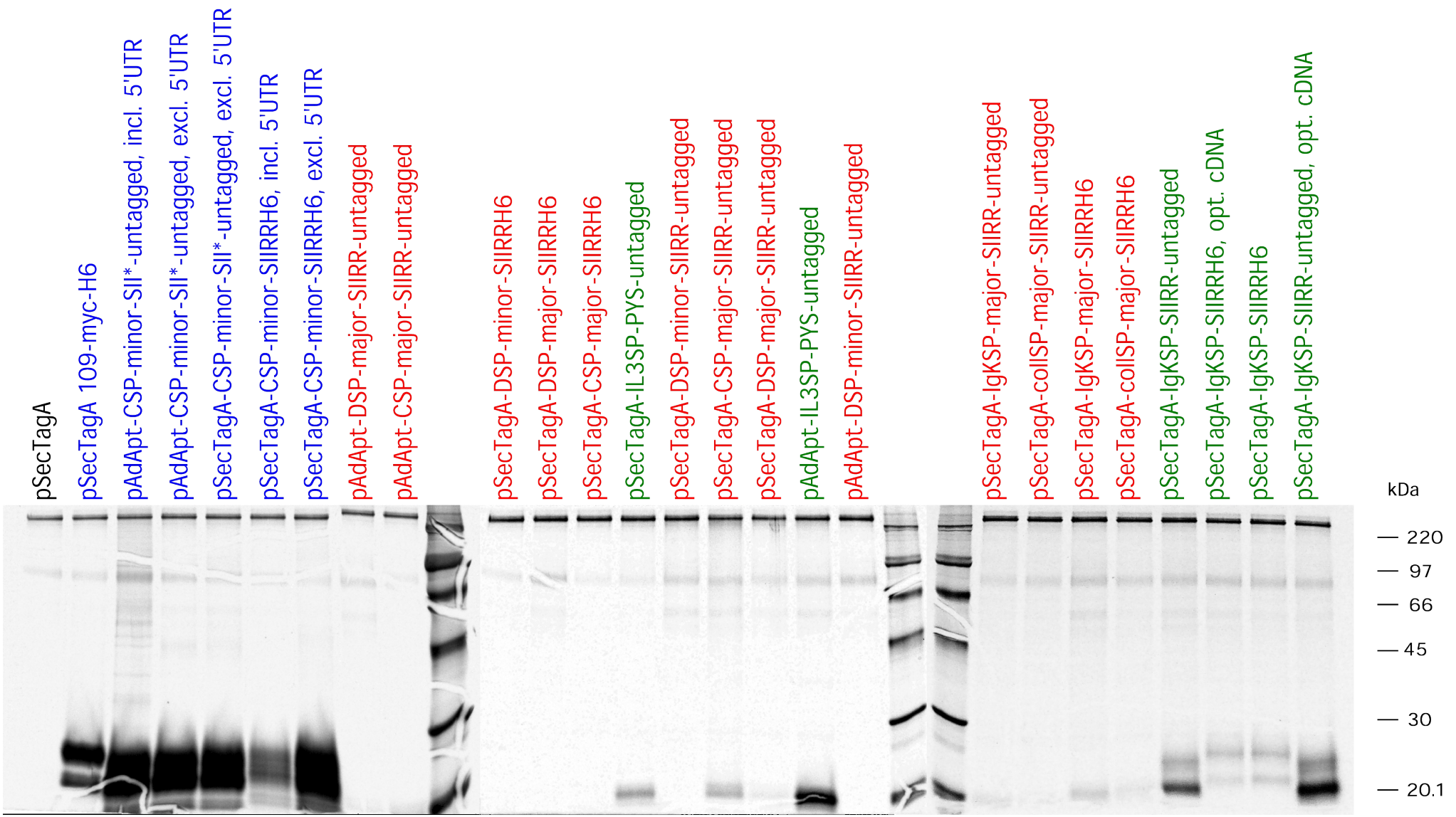
4. All mass preps must have a comparable quality (gel check, O/D ratio & concentration determination).

5. The comparison of expression levels should ideally be done all together to avoid experiment-to-experiment variation (alternatively a reliable internal cross-experimental control should be used).

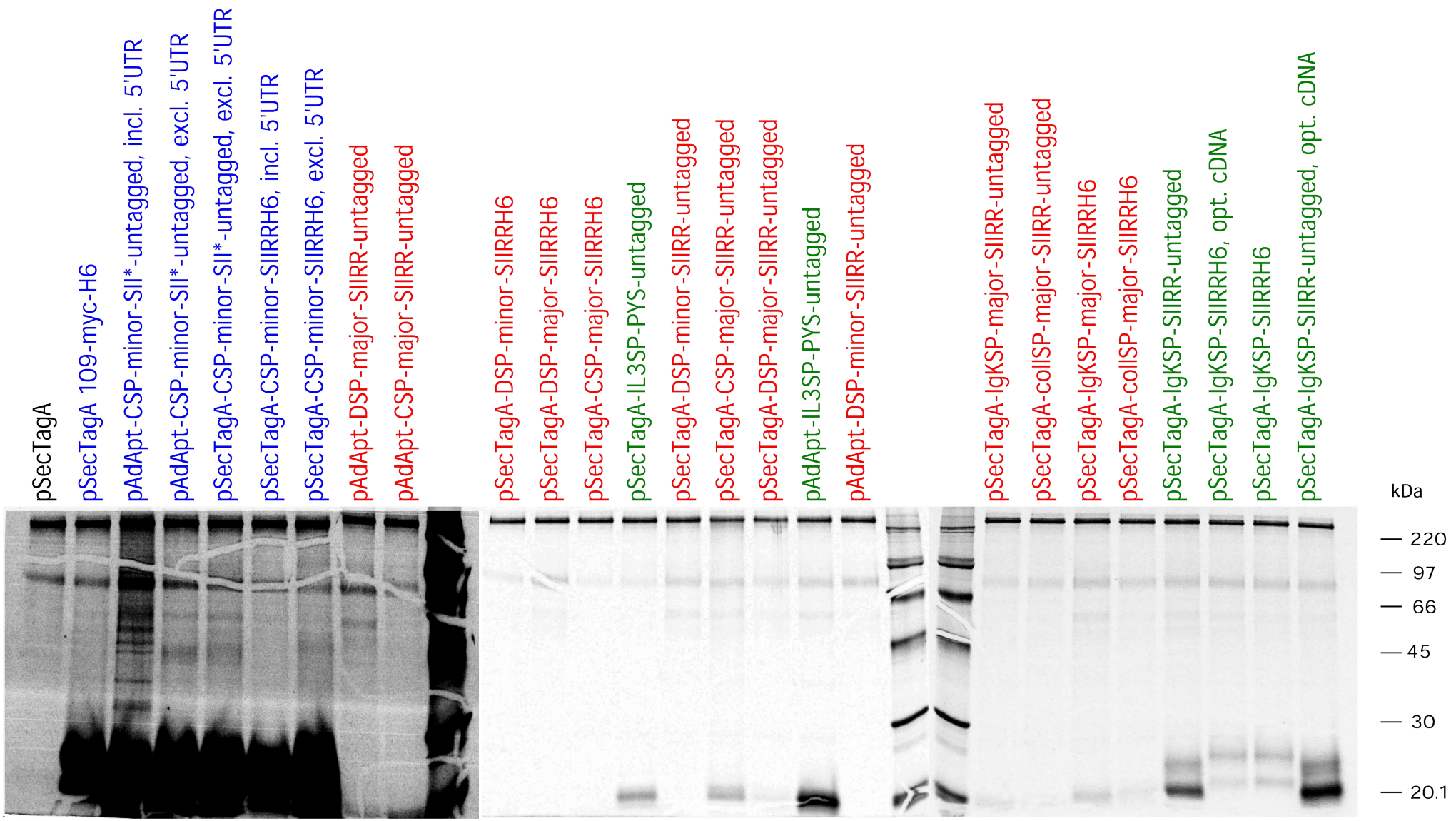
6. More potential pitfalls, but they were ignored (e.g. expression differences due to cell type).

Control constructs
pSecTagA-empty
pAdApt VEGF-C constructs (cloned by Marika Kärkkäinen)
pAdApt-CSP-ΔNΔC-hVEGF-Cminor_untagged, incl. 5'UTR
pAdApt-CSP-ΔNΔC-hVEGF-Cminor_untagged, excl. 5'UTR
VEGF-C CDS subcloned from pAdApt to pSecTagA
pSecTagA-CSP-ΔNΔC-hVEGF-Cminor-H6, incl. 5'UTR
pSecTagA-CSP-ΔNΔC-hVEGF-Cminor-H6, excl. 5'UTR
VEGF-C CDS subcloned from pAdApt to pSecTagA & histagged
pSecTagA-CSP-ΔNΔC-hVEGF-Dminor-H6, incl. 5'UTR
pSecTagA-CSP-ΔNΔC-hVEGF-Dminor-H6, excl. 5'UTR
pAdApt VEGF-D constructs (cloned by Tanja Laakkonen)
pAdApt-DSP-ΔNΔC-hVEGF-Dmajor_untagged
pAdApt-CSP-ΔNΔC-hVEGF-Dmajor_untagged
pAdApt-ΔNΔC-hVEGF-Dmajor_untagged
pAdApt-ΔNΔC-hVEGF-Dintermediate_untagged
VEGF-D CDS subcloned from pAdApt to pSecTagA
pSecTagA-DSP-ΔNΔC-hVEGF-Dmajor_untagged
pSecTagA-CSP-ΔNΔC-hVEGF-Dmajor_untagged
pSecTagA-ΔNΔC-hVEGF-Dmajor_untagged
pSecTagA-ΔNΔC-hVEGF-Dintermediate_untagged
VEGF-D CDS subcloned from pAdApt to pSecTagA & histagged
pSecTagA-DSP-ΔNΔC-hVEGF-Dmajor-H6
pSecTagA-CSP-ΔNΔC-hVEGF-Dmajor-H6
pSecTagA-ΔNΔC-hVEGF-Dmajor-H6
pSecTagA-ΔNΔC-hVEGF-Dintermediate-H6
VEGF-D constructs using three different signal peptides (cloned by Kukka Aho)
pSecTagA-IgKSP-hVEGF-Dmajor_untagged (9 N-terminal additional amino acids)
pSecTagA-IgKSP-SapI-hVEGF-Dmajor_untagged (native N-terminus predicted)
pSecTagA-collSP-hVEGF-Dmajor_untagged (native N-terminus predicted)
VEGF-D constructs using three different signal peptides, histagged
pSecTagA-IgKSP-hVEGF-Dmajor-H6 (9 N-terminal additional amino acids)
pSecTagA-IgKSP-SapI-hVEGF-Dmajor-H6 (native N-terminus predicted)
pSecTagA-collSP-hVEGF-Dmajor-H6 (native N-terminus predicted)
VEGF-D constructs with optimized codon usage & controls, tagged & untagged
pSecTagA-IgKSP-hVEGF-Dmajor_untagged (18 N-terminal additional amino acids)
pSecTagA-IgKSP-hVEGF-Dmajor-H6 (18 N-terminal additional amino acids), optimized cDNA
pSecTagA-IgKSP-hVEGF-Dmajor-H6 (18 N-terminal additional amino acids)
pSecTagA-IgKSP-hVEGF-Dmajor_untagged (18 N-terminal additional amino acids), optimized cDNA

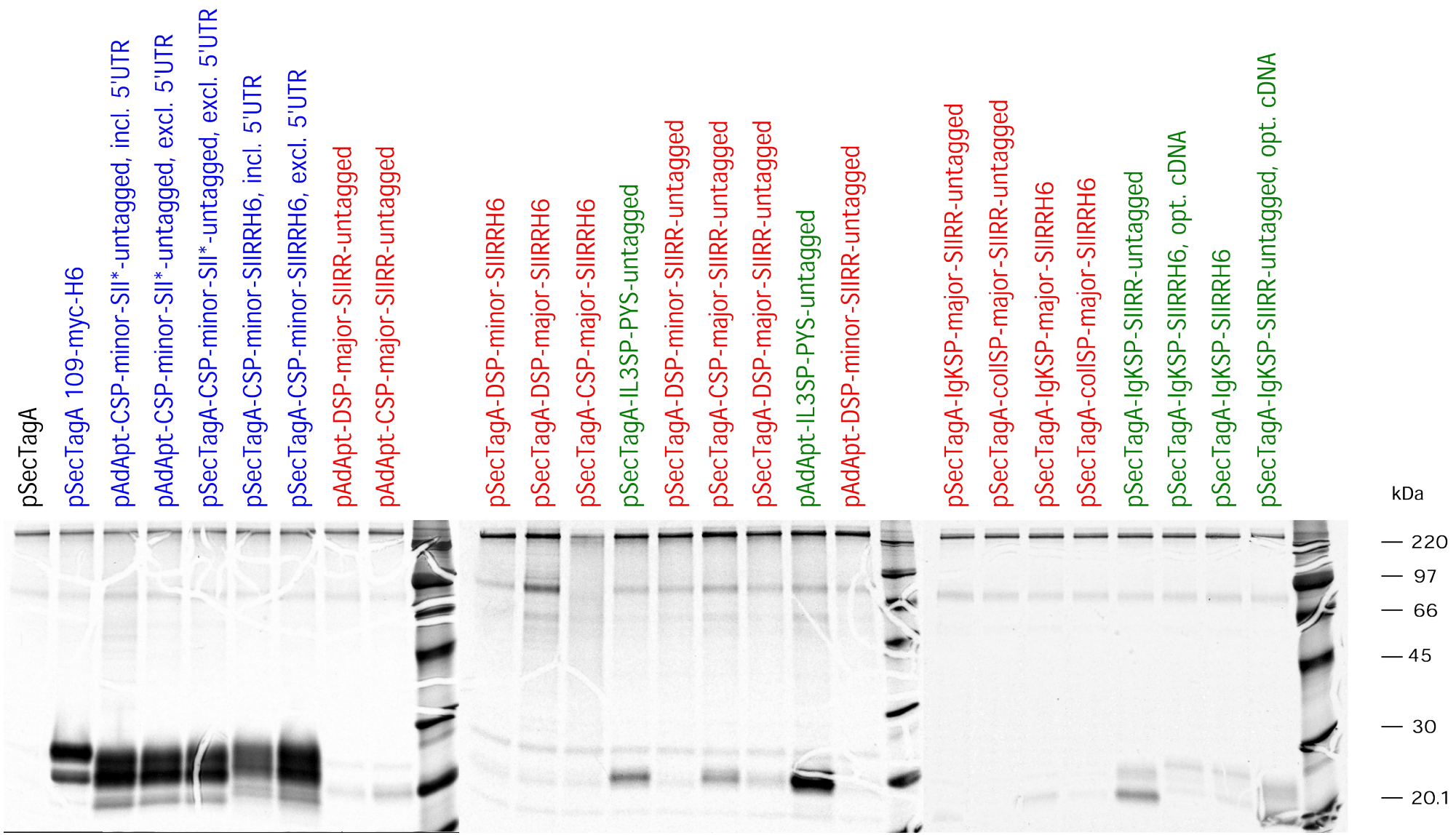
Binding of different mature VEGF-Ds to hVEGFR-3(D1-3)/hIgG1Fc



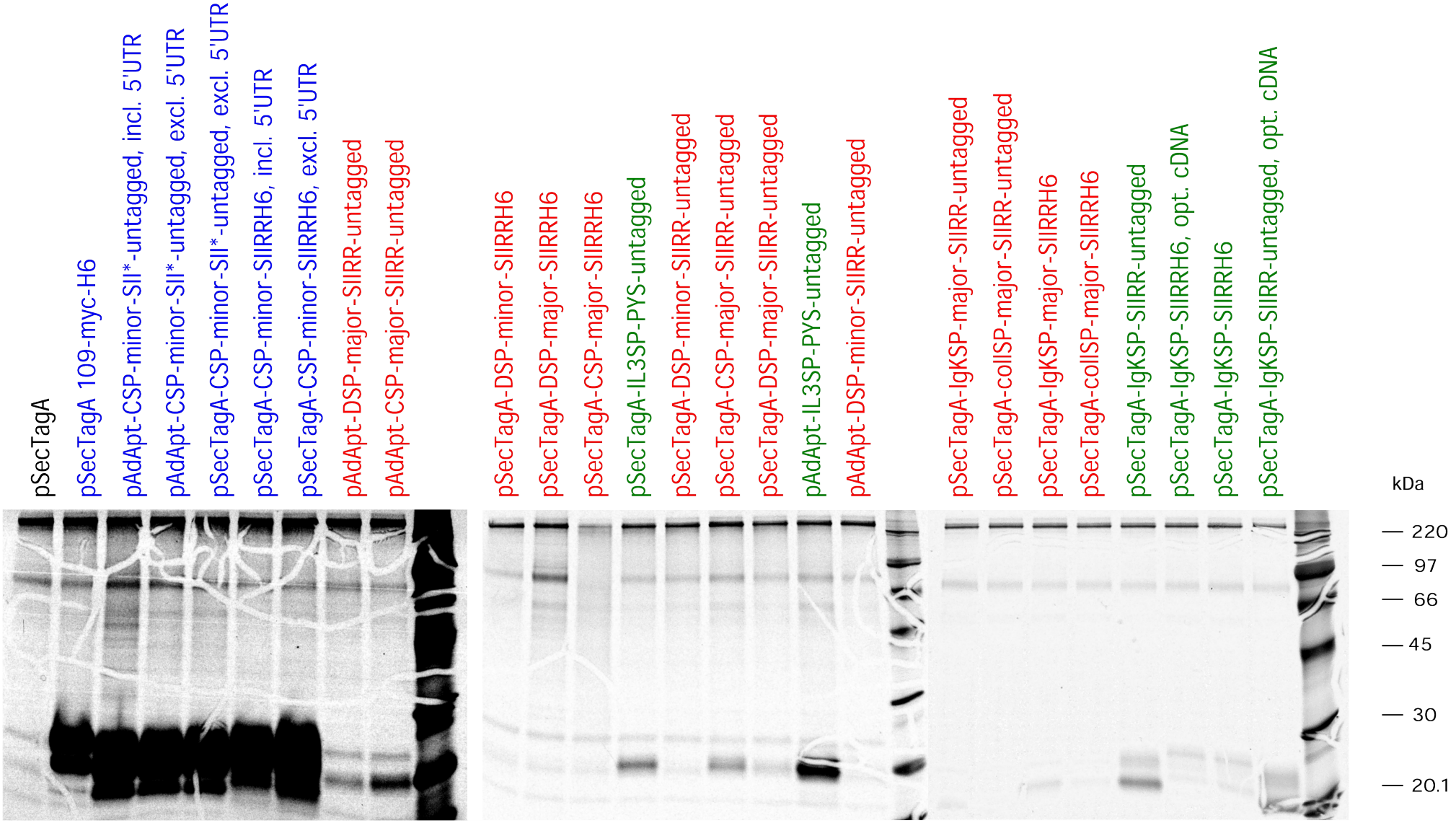
Binding of different mature VEGF-Ds to hVEGFR-3(D1-3)/hIgG1Fc



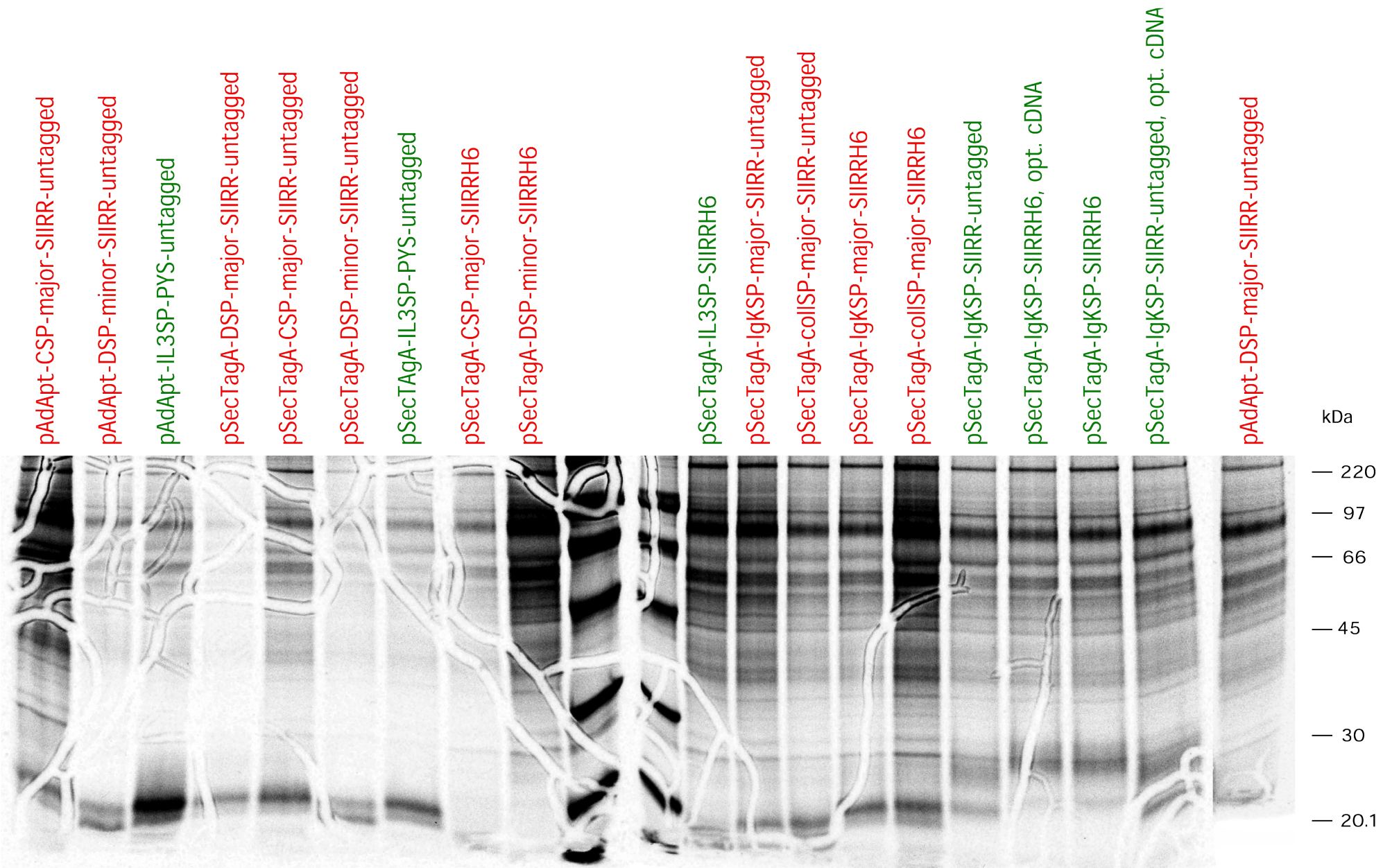
Binding of different mature VEGF-Ds to hVEGFR-2(D1-3)/hIgG1Fc



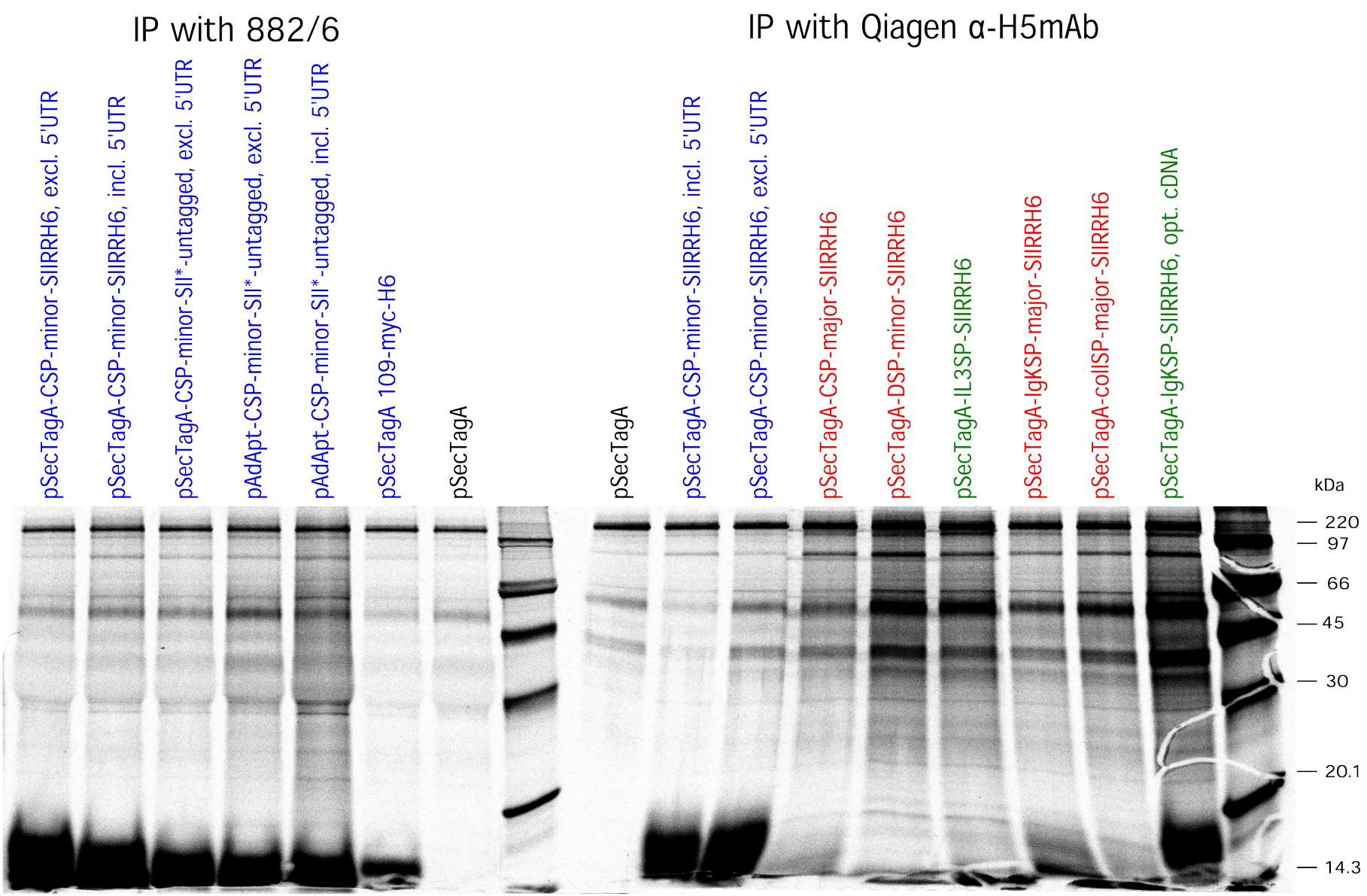
Binding of different mature VEGF-Ds to hVEGFR-2(D1-3)/hIgG1Fc



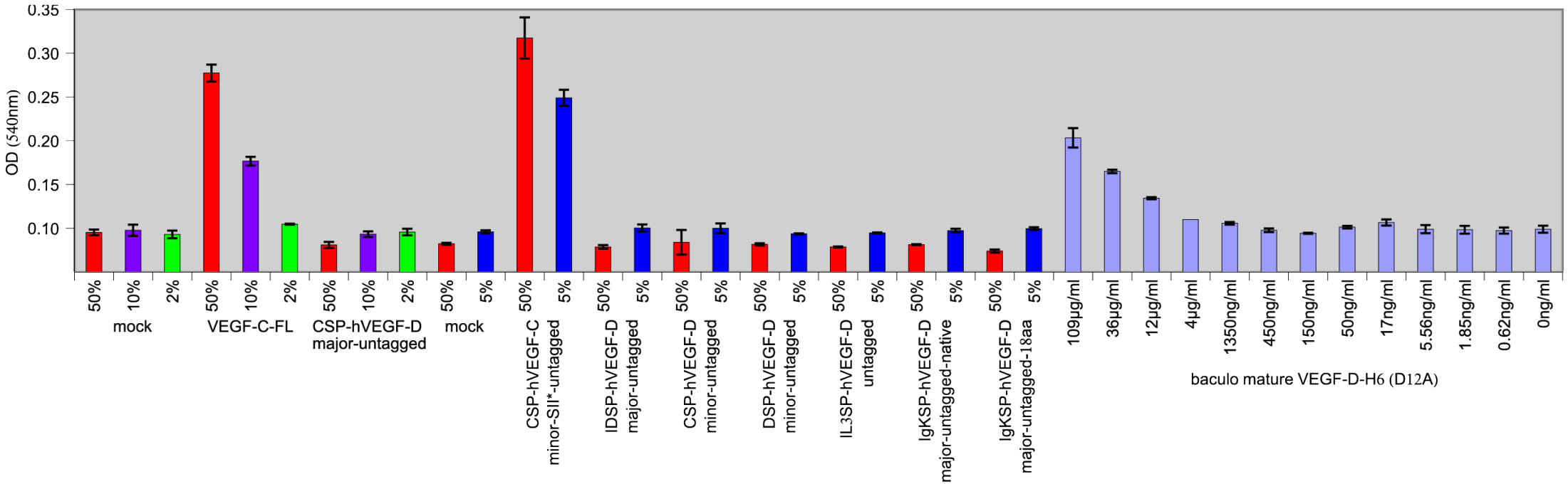
Expression of different mature VEGF-Ds (IP with pc α-VEGF-D)



Expression of different mature VEGF-Ds



Ba/F3-VEGFR-3 assay



Conclusions:

1. When comparing the expression levels of functional protein, none of the VEGF-D constructs came even close (>10%) to the expresion levels of the control VEGF-C constructs.

2. The constructs that expressed most VEGF-D were all non-native at their N-terminal ends (pSecTagA-IgKSP-SIIRRH6_opt._cDNA according to the H5mAb, pAdApt-IL3SP-PYS-untagged according to the VEGF-D pcAb and both VEGFR-2 and VEGFR-3).

3. Optimization of the cDNA doesn't increase the amount of functional protein. It might increase the amount of total protein.

4. None of the highest expressing VEGF-D constructs is suitable for use in humans.

5. From those constructs that were designed to be native at both termini, the one using the VEGF-C signal peptide had the highest expression.

6. Unlike for VEGF-C the usage of the C-terminal histag for mature VEGF-D is compromising binding towards both VEGFR-2 and VEGFR-3. Differently to Marika Kärkkäinen no difference could be seen between the VEGF-C constructs that differed by the absence/presence of the 5' UTR.

Production of various proteins

1. Recombinant production and purification of mature hVEGF-C in S2 cells for the selection of α-hVEGF-C scAb/α-hVEGF-C IgGAb

2. Recombinant production and purification of mature hVEGF-C in CHO cells

3. Characterization of new anti-VEGF-C antibodies (provided by VTT)

4. Recombinant production, purification and characterization of active hVEGFR-3/hIgGFc fusion protein in CHO cells

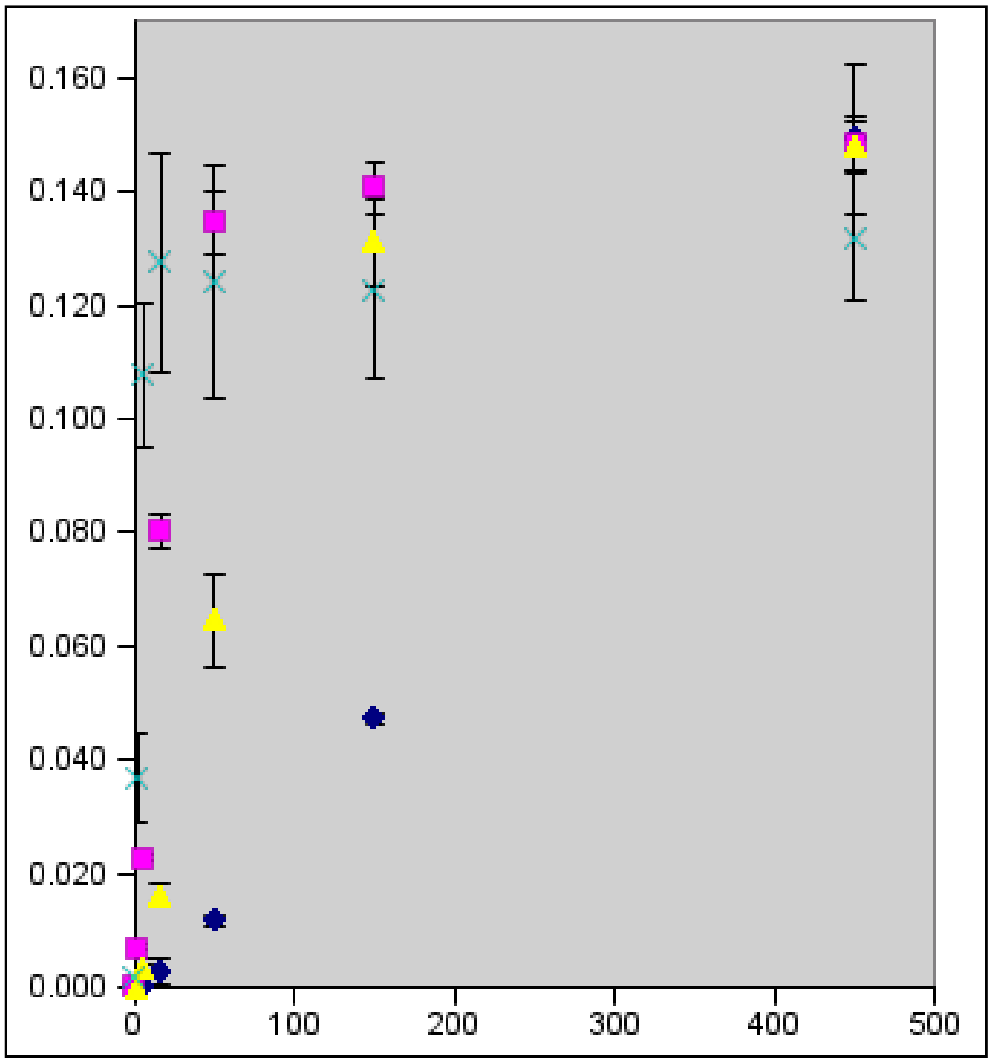
New recombinant VEGF-C proteins produced in S2 cells

1. New constructs:

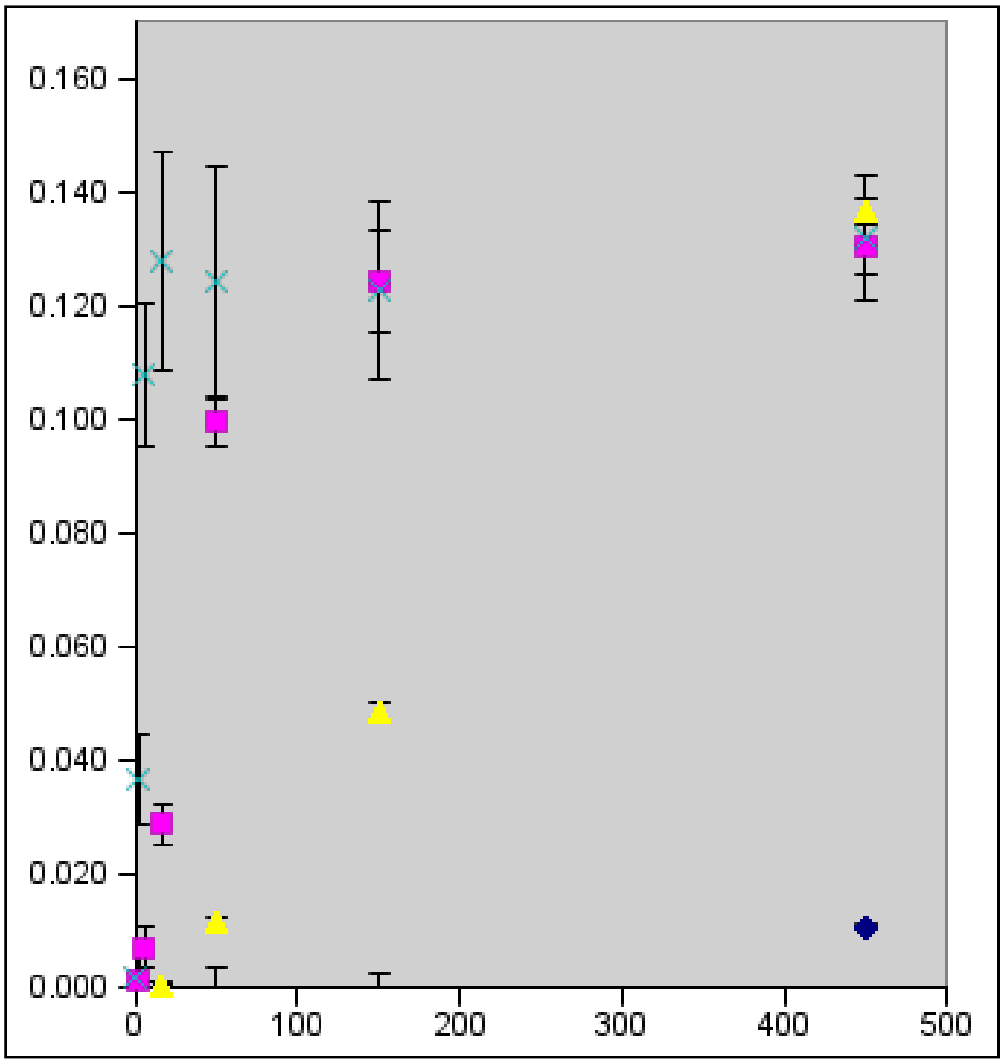
	DP-TEETIKFAAAHYN...	...MSKL-H6
minor form:	DP-TEETIKFAAAHYN...	...MSKLDVYRQVHSIIRR-H6
major form:	DP-AHYN...	...MSKLDVYRQVHSIIRR-H6

New recombinant VEGF-C proteins produced in S2 cells

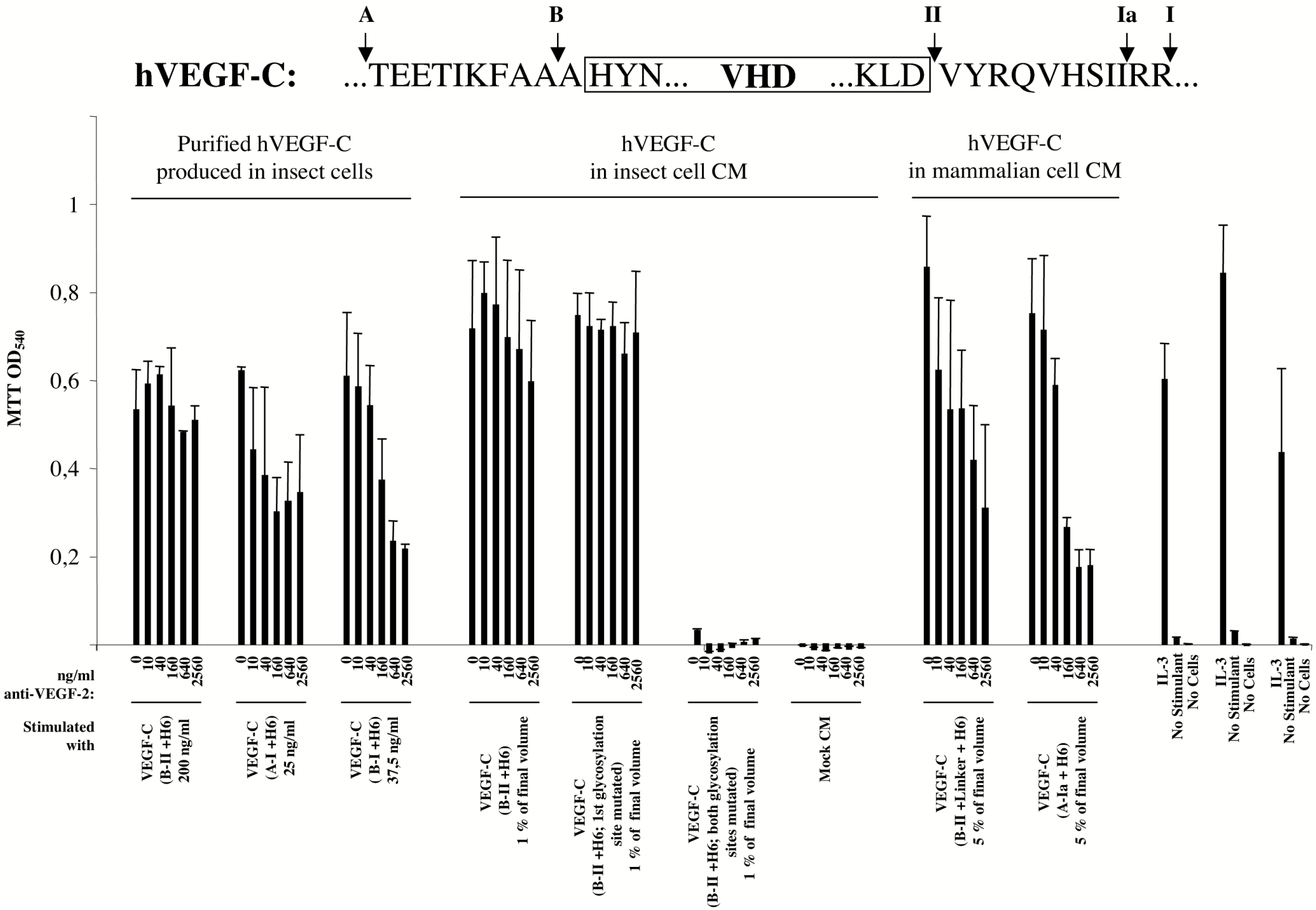
minor form

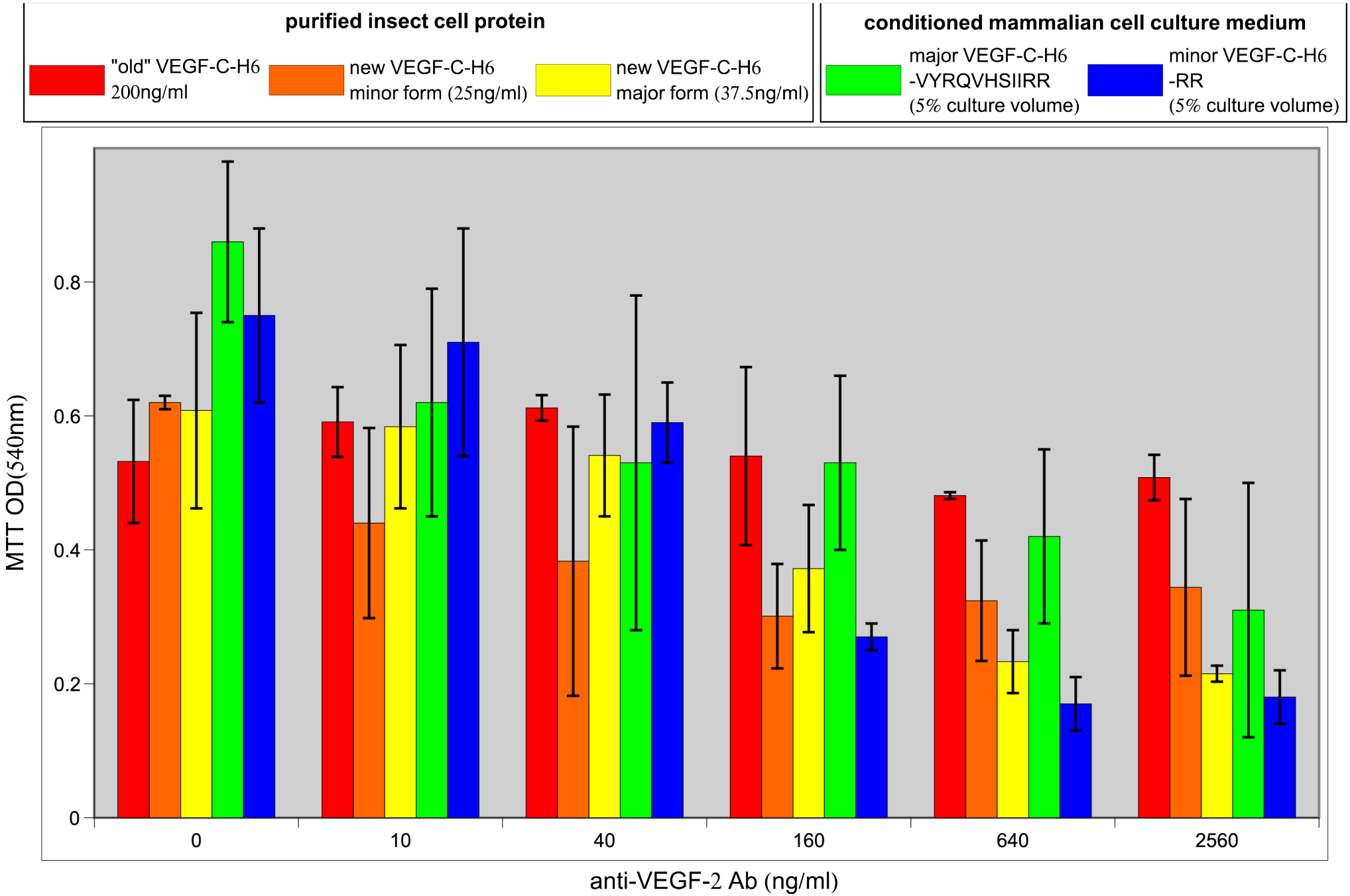


major form



blue = oligomeric/multimeric, magenta = dimeric, yellow = monomeric, cyan = "old VEGF-C standard"


A
B
II
Ia
I
hVEGF-C: ...TEETIKFAAA HYN... VHD ...KLD VYRQVHSIIRR...
Purified hVEGF-C produced in insect cells
hVEGF-C in insect cell CM
hVEGF-C in mammalian cell CM
MTT OD540
1
0,8
0,6
0,4
0,2
ng/ml
anti-VEGF-2:
0 10 40 160 640 2560
Stimulated with
VEGF-C (B-II +H6) 200 ng/ml
VEGF-C (A-I +H6) 25 ng/ml
VEGF-C (B-I +H6) 37,5 ng/ml
VEGF-C (B-II +H6) 1 % of final volume
VEGF-C (B-II +H6; 1st glycosylation site mutated) 1 % of final volume
VEGF-C (B-II +H6; both glycosylation sites mutated) 1 % of final volume
Mock CM
VEGF-C (B-II +Linker + H6) 5 % of final volume
VEGF-C (A-Ia + H6) 5 % of final volume
IL-3 No Stimulant No Cells
IL-3 No Stimulant No Cells
IL-3 No Stimulant No Cells


purified insect cell protein
"old" VEGF-C-H6 200ng/ml
new VEGF-C-H6 minor form (25ng/ml)
new VEGF-C-H6 major form (37.5ng/ml)
conditioned mammalian cell culture medium
major VEGF-C-H6 -VYRQVHSIIRR (5% culture volume)
minor VEGF-C-H6 -RR (5% culture volume)
MTT OD(540nm)
0
0.2
0.4
0.6
0.8
0
10
40
160
640
2560
anti-VEGF-2 Ab (ng/ml)

1. New constructs:

DP-TEETIKFAAAHYN... ...MSKL-*H6*

minor form: *DP*-TEETIKFAAAHYN... ...MSKLDVYRQVHSIIRR-*H6*

major form: *DP*-AHYN... ...MSKLDVYRQVHSIIRR-*H6*

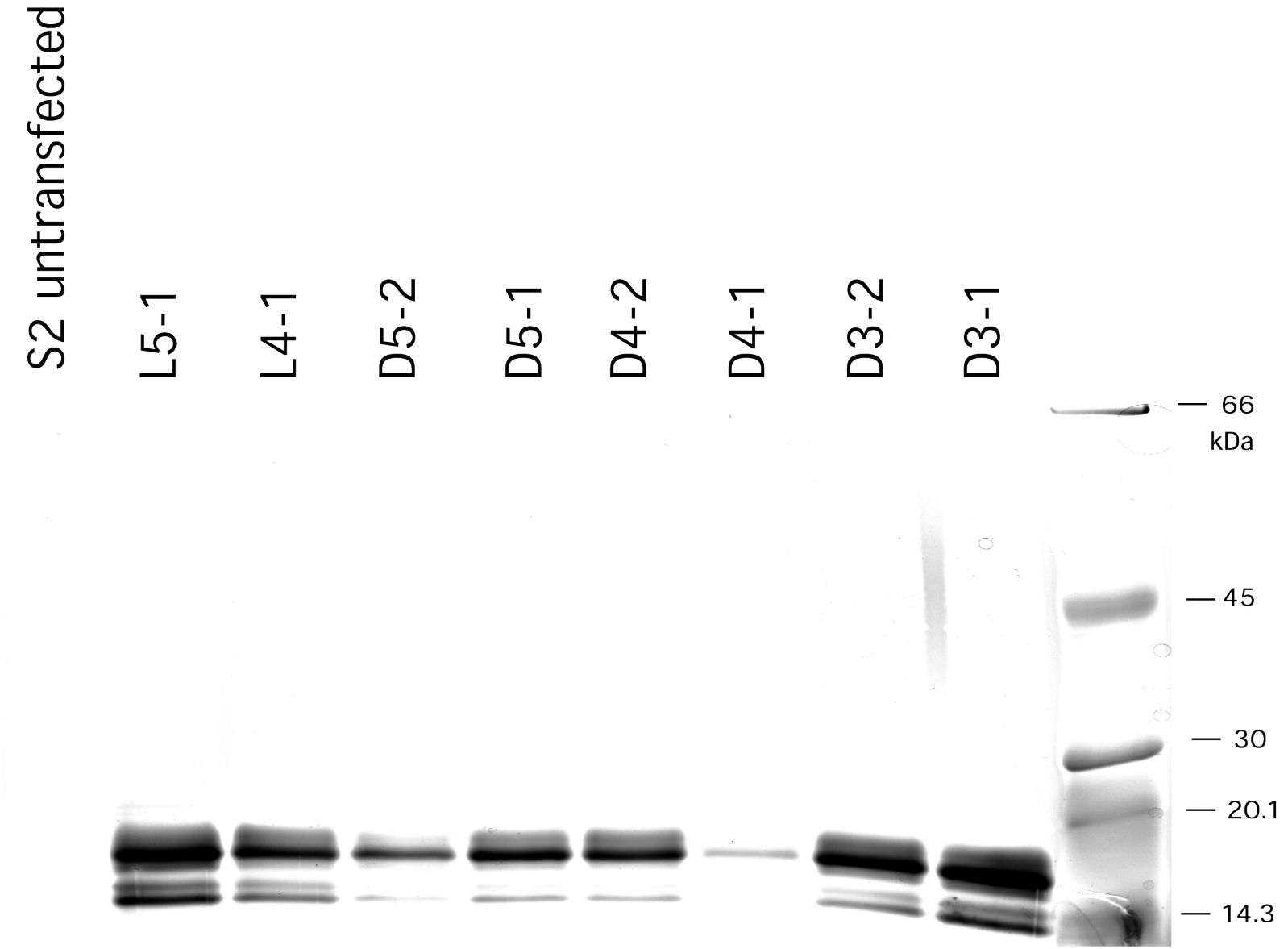
2. Modified purification procedure:

Complete separation of dimeric form from mono- and oligomeric forms via a HiLoad 26/60 Superdex 75 column.

3. Different tags for the minimal form:

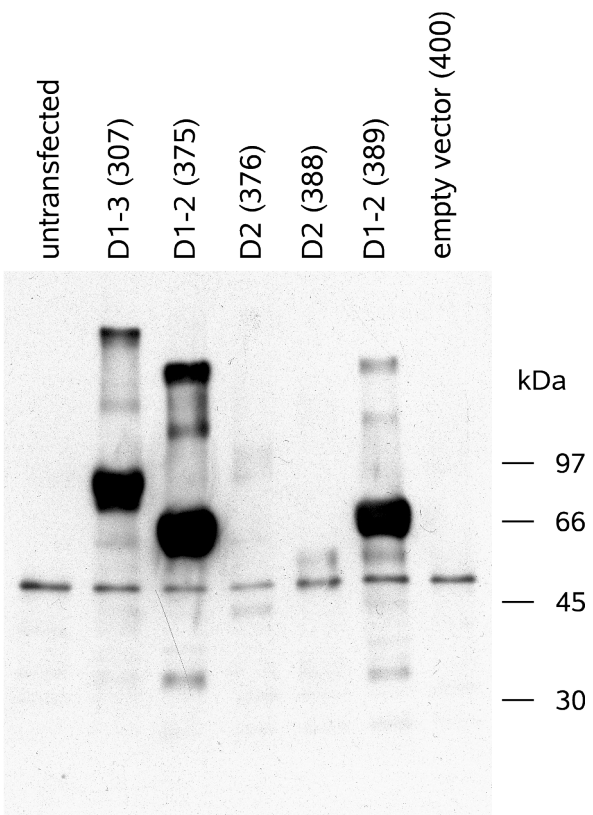
CBU-tag: AHYN... ...MSKL-*KRRWKKNFIAVSAANRFKKISSSGAL*
StrepII-tag: AHYN... ...MSKLD-SA-*WSHPQFEK*
(StrepIII-tag: AHYN... ...MSKLD-SA-*WSHPQFEK*-GGGSGGGSGGGS-*WSHPQFEK)*

minimal VEGF-C-CBU
(calmodulin-sepharose & elution with 2 mM EGTA)

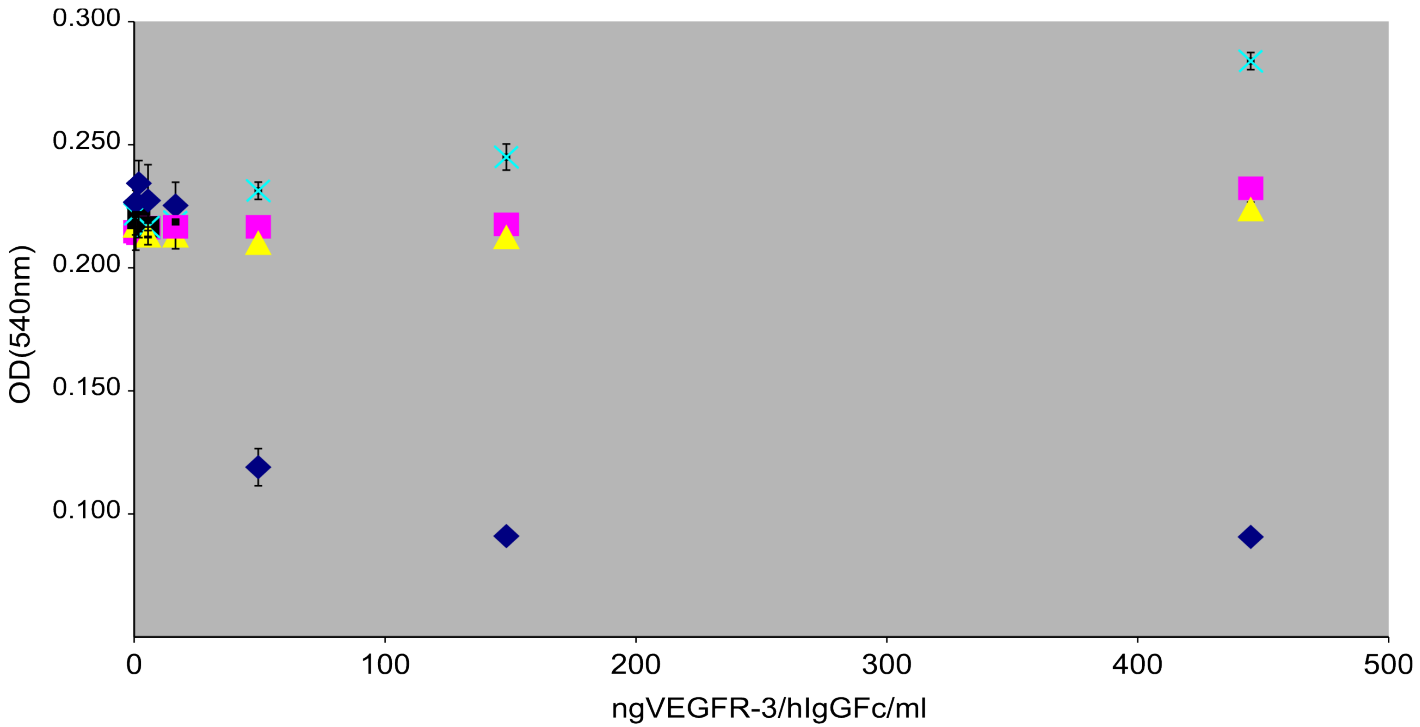


Comparison of VEGFR-3(D1-3)/hIgGFc and VEGFR-3(D1-2)/hIgGFc

Western with α-hIg/HRP



Inhibition of VEGF-C-mediated survival of Ba/F3-VEGFR-3 cells (45ng VEGF-C/ml)



Production of recombinant proteins in mammalian hosts

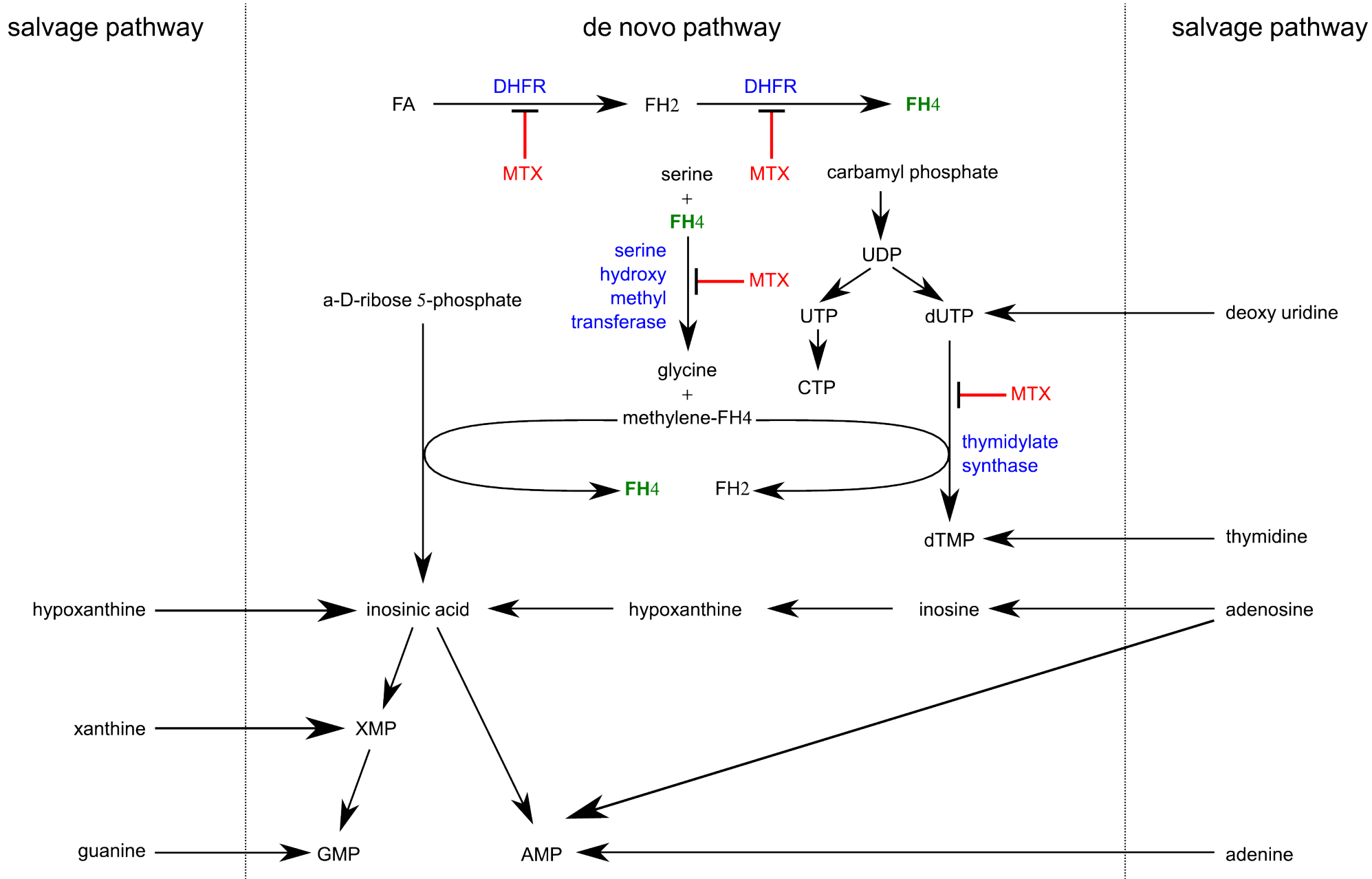
typical product titre: ~50mg/l (1986) vs. 4.7g/l (2004)

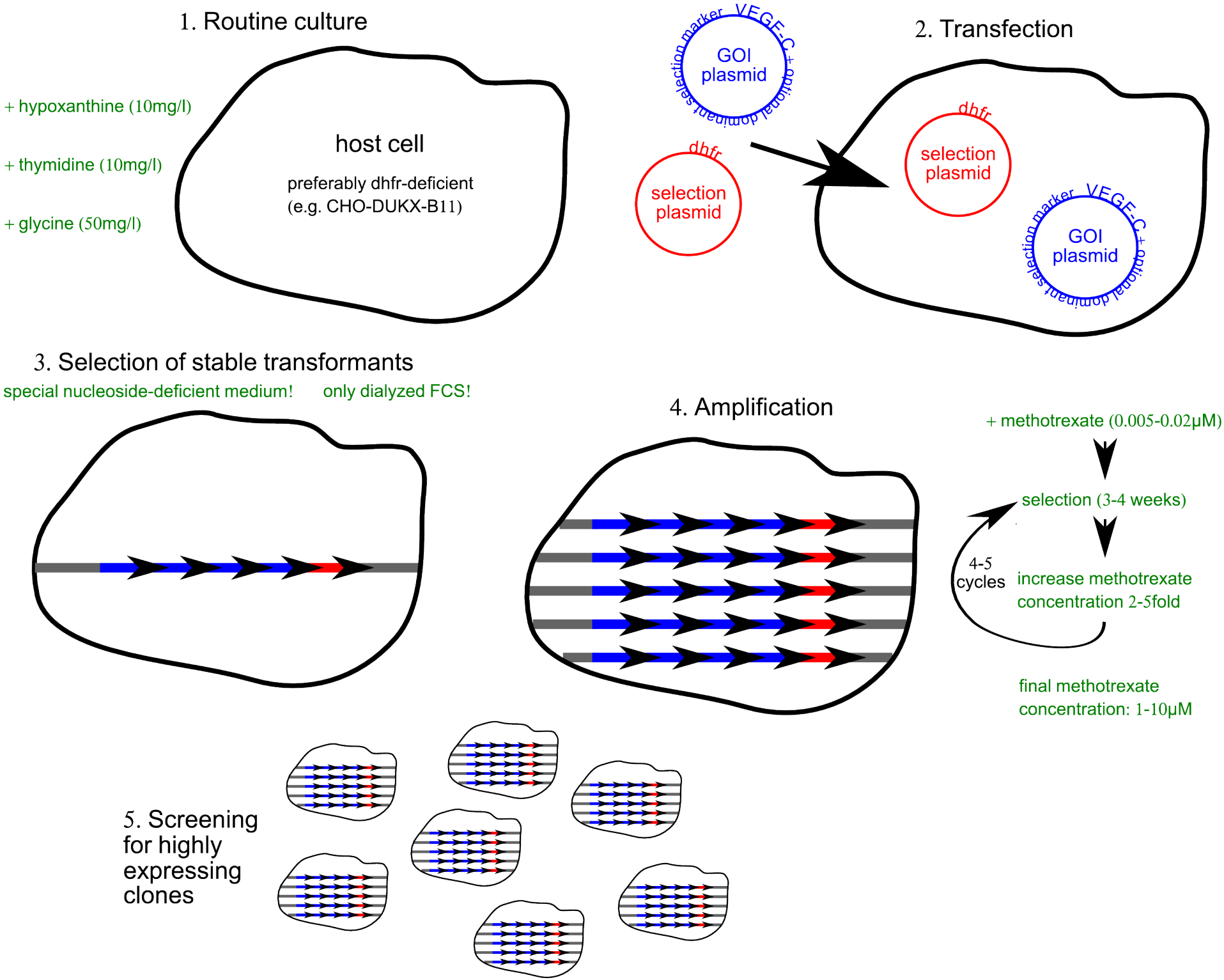
sources of improvement:

cell density (4-5x higher)
medium & fermentation optimization (roller-bottles vs. fermenter), host cell engineering

production phase (2-3x longer)
host cell engineering, fermentation optimization

specific productivity (5-10x more)
HT screening, host-cell engineering (site-specific integration)


salvage pathway
de novo pathway
salvage pathway
FA
DHFR
FH2
DHFR
FH4
MTX
MTX
serine
+
FH4
carbamyl phosphate
serine
hydroxy
methyl
transferase
MTX
UDP
a-D-ribose 5-phosphate
UTP
dUTP
deoxy uridine
glycine
+
CTP
MTX
methylene-FH4
thymidylate
synthase
FH4
FH2
dTMP
thymidine
hypoxanthine
inosinic acid
hypoxanthine
inosine
adenosine
xanthine
XMP
guanine
GMP
AMP
adenine


1. Routine culture
+ hypoxanthine (10mg/l)
+ thymidine (10mg/l)
+ glycine (50mg/l)
host cell
preferably dhfr-deficient
(e.g. CHO-DUKX-B11)
2. Transfection
GOI plasmid
selection marker
VEGF-C + optional dominant
dhfr
selection plasmid
3. Selection of stable transformants
special nucleoside-deficient medium!
only dialyzed FCS!
4. Amplification
+ methotrexate (0.005-0.02µM)
selection (3-4 weeks)
4-5 cycles
increase methotrexate concentration 2-5fold
final methotrexate concentration: 1-10µM
5. Screening for highly expressing clones

Major Challenges of dhfr Gene Amplification in Chinese Hamster Ovary (CHO) Cells

1. During selection and amplification low-expressing clones are preferentially selected (inverse relationship between protein expression levels and doubling time

2. Resistance against methotrexate (MTX) does occur due to alternative drug-resistance mechanisms, especially under rapid selection

3. Screening needs a HT approach find highly expressing clones from the tail of the Gauss curve

4. Protein expression levels are inversely related to protein quality

Major Challenges of dhfr Gene Amplification in Chinese Hamster Ovary (CHO) Cells

1. During selection and amplification low-expressing clones are preferentially selected (inverse relationship between protein expression levels and doubling time)

- Early and multiple rounds of single-cell expression screening
- Expression feedback (e.g. expressing the selection marker as a fusion protein with a EC domain capable of autocrine survival signalling)

2. Resistance against methotrexate (MTX) does occur due to alternative drug-resistance mechanisms, especially under rapid selection

- Early and multiple rounds of single-cell expression screening
- Slow selection

3. Finding a highly expressing clone from the tail of the Gauss curve

- targeted insertion
-HT screening

4. Protein expression levels are inversely related to protein quality

-engineering of host cells (enzymes of the glycosylation machinery, chaperones)

1. pSV2-dhfr

2. pSV2-dhfr^{weak} and pSV2-dhfr^{weakweak} (two additonal versions of pSV2-dhfr with a weakly and a heavily crippeled SV40 promoter)

3. pSV2-dhfr/EGFP

(4. pSV2-dhfr^{weak}/EGFP and pSV2-dhfr^{weakweak}/EGFP)

5. Insertion of the VEGFR-3/hIgGFc and VEGF-C expression casettes into the selection vector (alternative but inferior for CHO cells: cotransfection with existing constructs)

Acknowledgements

Kukka Aho

Kari Alitalo

Terhi Kärpänen

Tanja Laakkonen

Tuomas Tammela