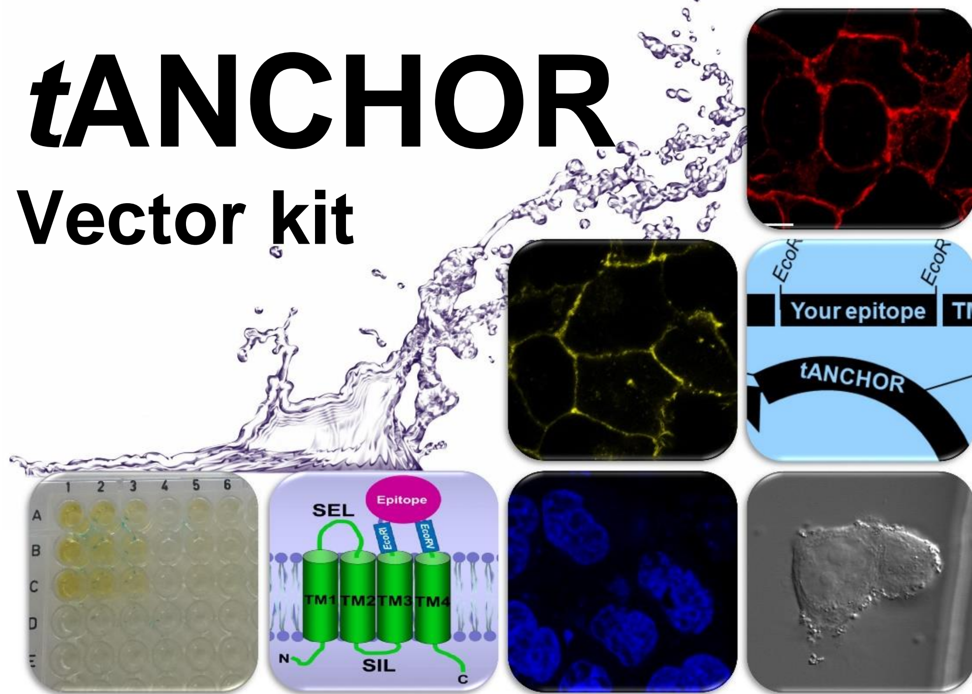
*t*ANCHOR Vector kit



For presenting peptides on the surface of mammalian cells

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This kit is for research only!

Not for diagnostic purposes!

This product requires knowledge of basic molecular biological techniques

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Data Safety and Confidentiality Statement:

ATG:biosynthetics declares that we will keep your intellectual property about your genes/ sequences in strict confidentiality and we will NOT, in any manner, disclose, develop and utilize your genes/ sequences. We will destroy all the related testing data and results after at least one month of storing. On request we will provide a secure web space for mutual data exchange.

Security Statement:

Please note that ATG like other gene synthesis companies in compliance with bio-security relevant self-control has subjected itself to a “code of conduct” which is emerging to be an international quality management standard of the world wide community of Synthetic Biologists. Sequences are checked for their potential of being a bio-hazard or their potential for misuse in bio-terrorism. If you know that your gene has any such potential, please cooperate with the manufacturer or distributor, e.g. ATG.

By buying this product you are accepting the license conditions and agree.

Otherwise you will send immediately back all items of the *t*ANCHOR kit without keeping items back or share it with third parties.

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1. Product information

Content: 5 µg of each *t*ANCHOR vector (lyophilized in TE buffer)

Storage: Long term: lyophilized vectors should be stored at -20°C
Short term: TE buffer 4°C or in distilled water at -20°C

Product characterization: *t*ANCHOR vectors can be characterized by restriction enzymes showing the correct following banding pattern

Vector	Enzyme	Fragment length in bp
*t*ANCHOR-CD63	*Nhe*I/*Cla*I	621 and 3535
*t*ANCHOR-CD63	*Xho*I/*Eco*RI	1098 and 3058
*t*ANCHOR-CD82	*Nhe*I/*Cla*I	666 and 3535
*t*ANCHOR-CD82	*Xho*I/*Eco*RI	1113 and 3088

License number: Each purchased *t*ANCHOR kit will be shipped with a unique license number. The license number is stamped on the *t*ANCHOR kit package. Please keep the license number (customer#) for your records, it will enable you to receive quick and personalized support. Please consult us if you encounter any problems during your applications with the *t*ANCHOR vectors. We will help you as quickly as possible.

2. Introduction

The *t*ANCHOR technology provides an innovative and versatile tool for displaying epitopes on the surface of e.g. human cells which was developed by Dr. Daniel Ivanusic. This system is based on the use of tetraspanin anchors (*t*ANCHOR). Transmembrane anchors derived from a bundle of 4 tetraspanin transmembrane domains assembled as a type III integral membrane protein are highly efficient to transport epitopes to the surface of human cells. The main characteristics of tetraspanin protein family consist of the presence of a small and a large extracellular loop (SEL and LEL) which are flanked by four transmembrane domains. The epitope sequence for displaying on the cell surface is placed instead of the large extracellular loop of the tetraspanin full-length sequence and is flanked by the restriction sites *Eco*RI and *Eco*RV (**Fig. 1**).

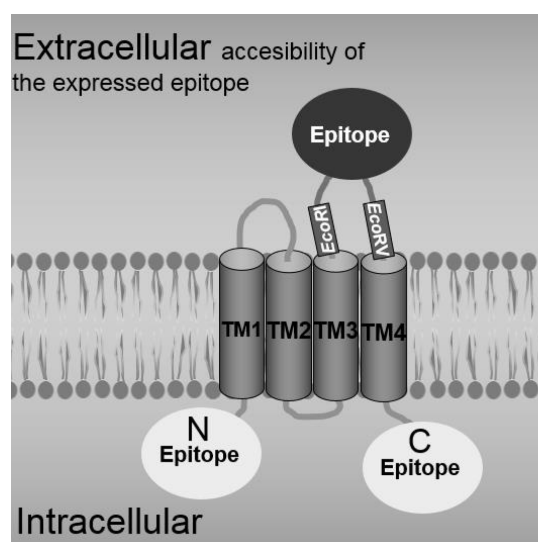


Fig. 1

2.1 Application

During its development the *t*ANCHOR system was verified and tested for functionality by displaying epitopes 2F5-4E10 derived from HIV-1, V5-6xHis, relevant epitopes for checkpoint targeted therapies of human PD-1 and CD20 on the cell surface. Confocal analysis of *t*ANCHOR proteins fused to mCherry (**Fig. 2**) showed correctly expression on the surface of human cells (HEK293T, HeLa). The extracellular orientation was tested with anti-V5 in an ELISA based assay and showed that epitopes are highly extracellular accessible. Expresssed *t*ANCHOR proteins can be used for bioanalytical methods (e.g. ELISA), protein-

protein-interaction studies on the cell surface or as antigen expression system for immunization studies.

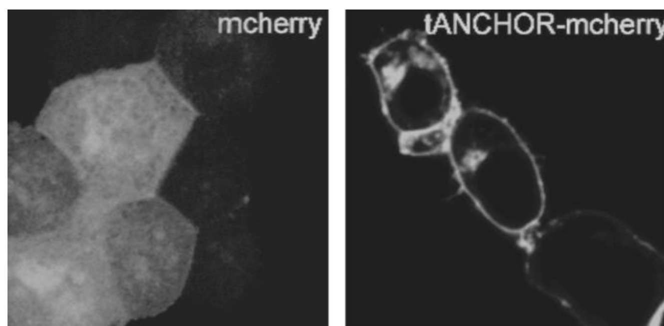
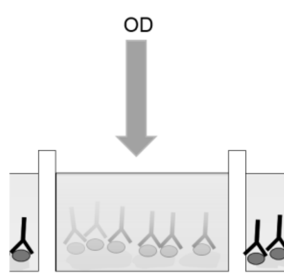


Fig. 2

We recommend that the constructs should be fused C-terminal with a mCherry fusion-tag. This fusion will significantly reduce the time needed for localization verification of expressed proteins. Delivered *t*ANCHOR vectors do not contain fusion of a mCherry sequence.

*t*ANCHOR technology provides an innovative tool for displaying epitopes on the surface of human cells. This enables you to improve your ELISA methods to search for antibodies binding studies under native conditions on your target (**Fig. 3**).



1. Seed cells
2. Transfect and incubate
3. Antibody incubation
4. Washing steps
5. HRP detection (TMB)

Fig. 3

<u>The benefits are:</u>

-Maximize your search for antibodies under native conditions
-No need to coat plates with expensive peptides
-High throughput screening: seed and transfect cells – incubate your antibodies
-Fast and reliable results

3. Methods

3.1. Using the right *t*ANCHOR vector

The *t*ANCHOR FULL kit contains two *t*ANCHOR ASSAY-vectors *t*ANCHOR-CD63 and tANCHOR-CD82 and 6 control vectors. We recommended start with p*t*ANCHOR-CD82 if you are using HeLa as target cells. If you are using cells where endogenous CD63 is highly expressed, then use *t*ANCHOR-CD63.

3.2. Cloning of epitope sequences in the *t*ANCHOR system

Upon receiving the vial resuspend the lyophilized *t*ANCHOR vectors in 50 µl sterile water to make a 0.1 µg/µl stock solution. Vortex for 1 minute and store the stock solution at -20°C. Use this stock solution to transform an *E. coli* strain such as TOP10 or DH5alpha. If you are using other *E. coli* strains, ensure that they are recombination (recA) and endonuclease A deficient (endA). The *t*ANCHOR vector system is a fusion protein expression vector requiring that you have to clone your epitope sequence in frame between restriction sites *Eco*RI and *Eco*RV with the N- and the C-terminal part of the tetraspanin transmebrane sequence (**Fig. 4**). See sequence map and cloning diagram for designing your cloning strategy (**Fig. 5-7**). Please consult us at support@atg-biosynthetics.com if you have further questions. The sequences of *t*ANCHOR vectors are delivered with the *t*ANCHOR kit or are available upon request.

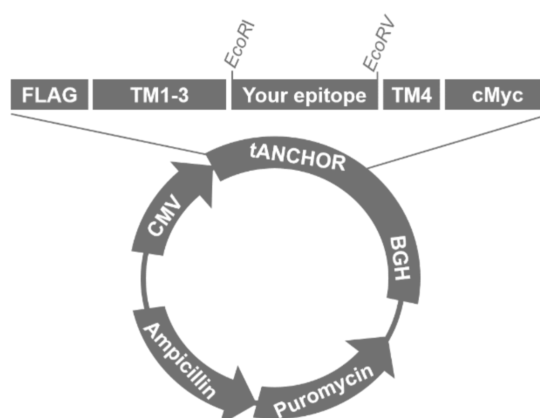


Fig. 4

3.3. Vector features

The *t*ANCHOR vectors contains the following feature elements.

Feature	Restriction sites	Function	Position	
			ptANCHOR-CD63	ptANCHOR-CD82
***Nco*I-X-*Nco*I**	CC|AT|GG	**FLAG**-tag	732-740	732-740
***BstX*I**	CCAN|NNNN|NTGG	Alternative site for introduction of **5'-tags**	762-773	762-773
***Nco*I-*EcoR*I**	CC|AT|GG —G|AATT|C	**TM1-3**	761-1105	762-1115
***EcoR*I-*EcoR*V**	G|AATT|C — GAT||ATC	**V5-6xHis-**tag control epitopes	1100-1201	1115-1216
***EcoR*V-*Cla*I**	GAT||ATC — AT|CG|AT	**TM4**	1196-1339	1211-1384
***Cla*I-*Pme*I**	AT|CG|AT — GTTT||AAAC	**c-myc**-Tag control epitope	1334-1377	1384-1422
***Pme*I**	GTTT|AAAC	Remove **empty** vectors	1370-1377	1415-1422

Table 1

3.4. Vector maps

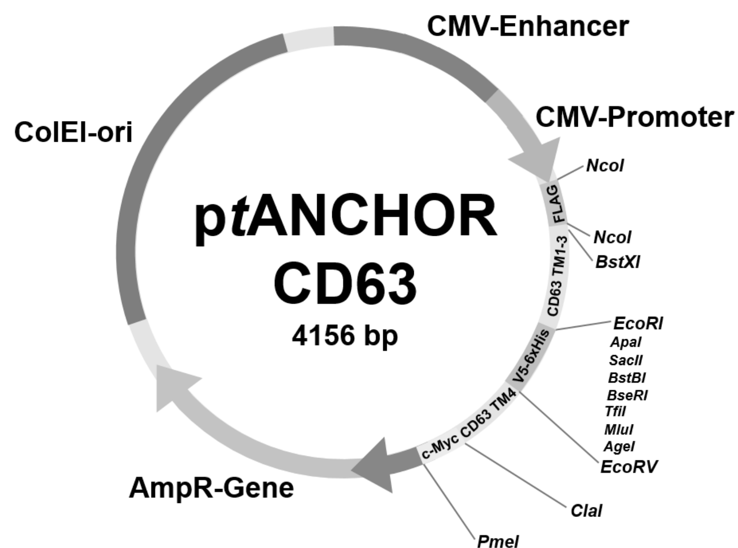


Fig. 5

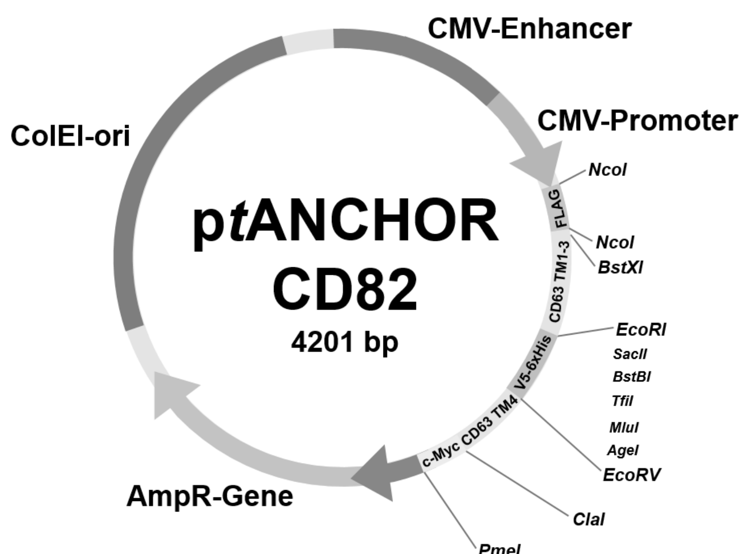


Fig. 6

In order to introduce your epitope sequence in the *t*ANCHOR vector create a fragment by PCR or gene synthesis with the flanked by the sequence of appropriate restriction sites GAATTC (*EcoR*I) and GATATC (*EcoR*V). Clone these fragments using standard cloning procedures. Note: in order to cut effectively 4-6 nucleotides should be added on either side of the recognition site. Detailed information is available from the restriction enzyme supplier like on the homepage of New England Biolabs (Cleavage Close to the End of DNA Fragments). After incubation of your ligation batch over night at 16°C transform into chemically competent *E. coli* cells, plate cells on LB agar plates containing 100 µg/ml Ampicillin, pick clones and check recovered plasmids for the presence of the insert of interest using restriction enzymes and confirm sequence by Sanger DNA sequencing. In order to keep the rate of empty vectors low you can incubate your ligation batch with *Pme*I.

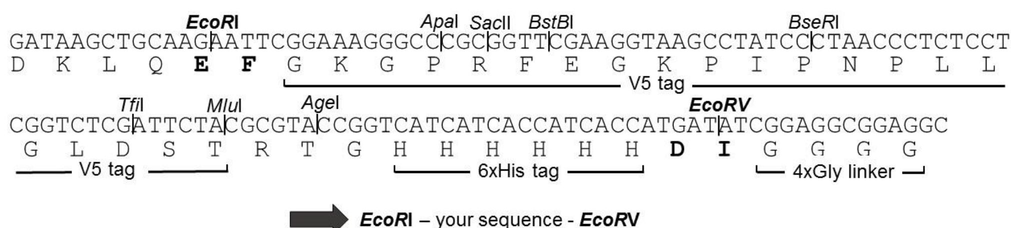
Use the following sequence primers to confirm your construct:

	Primer sequence 5´-3´
Seq1_for	GGATAGCGGTTTGACTCACG
Seq1_rev	GCACAGTCGAGGCTTTATGT

3.5. Cloning diagram

Carefully inspect your cloning strategy with the following map. Your epitope sequence must be *in frame* with the *t*ANCHOR sequence as shown in the cloning diagram. The control vector contains a V5-6xHis insert which is in frame between the *EcoR*I and *EcoR*V restrictions sites restriction sites and serves as an example for the cloning strategy. We recommend inserting your fragment between the *EcoR*I/*EcoR*V restriction sites. The V5 or 6xHis epitope can be fused with your epitope sequence by inserting your sequences between *EcoR*I/*Age*I or *Age*I/*EcoR*V.

tANCHOR-CD63



tANCHOR-CD82

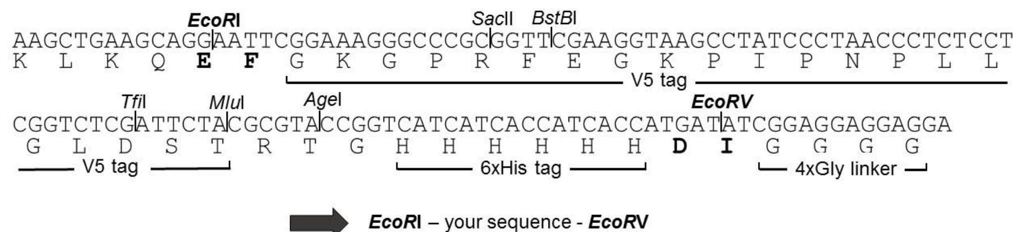


Fig. 7

3.6. Transfection of human cells

This section is provided for your convenience to increase research success. More details are provided in the references.

Once the correct sequence of your clone is confirmed you will have to prepare plasmid DNA for transfection of cells. Isolated plasmid DNA for transfection into human cells have to be highly pure and containing low endotoxin (LPS) contaminations. LPS contaminations are able to kill cells or decrease

transfection efficiency. Inpurities with salt will in addition lead to decrease your transfection efficiency. We recommended to prepare plasmid DNA using the EndoFree MaxiPrep kit from Qiagen, Germany. For transfection of HEK293T or HeLa cells we recommended MetafectenePro from Biontex, Germany for high transfection efficiency of *t*ANCHOR vectors according to manufacturer's information. Jurkat cells (immortalized cell line of T lymphocyte cells) were also tested using AMAXA transfection kit with the nucleofection program S-10. Insertion of mCherry (see vector map for cloning strategy) instead of the cMyc tag will easy display ease display transfection efficiency. The use of a transfection control e.g. pmCherry-N1 from Clontech, Germany is useful.

3.7. Verification of protein expression

Verify your protein expression by Western blot analysis from total cell extracts using either an antibody directed against your inserted epitope of interest or an antibody directed against the N-terminal FLAG-tag or C-terminal cMyc-tag of the fusion protein. After confirming a positive protein expression proceed with the protein localization analysis.

3.8. Detection of displayed epitopes

Basic *t*ANCHOR vectors does not include a fluorescence reporter protein e.g. mCherry therefore displayed epitopes have to be visualized by indirect immunofluorescence technique. We recommended to detect the V5 epitope in the basic vector with an anti-V5 antibody and a secondary fluorescently conjugated antibody. Instead of detecting the extracellular orientation by indirect immunofluorescence there is also the possibility to test by standard ELISA-based methods. For this application seed cells in a 96 well plate, transfect and test the indicated presented peptide by an antibody recognizing an epitope within the peptide (**Fig. 3**). The bound antibody can be detected an HRP-conjugated secondary antibody. Use a control antibody e.g. anti-cMyc for excluding intracellular or unspecific antibody binding.

4. Troubleshooting

Issue	Possible reason	Solution
No protein expression (western blot analysis)	Low transfection efficiency	Improve transfection efficiency
	Low DNA quality	Prepare new plasmid DNA batch
	Epitope sequence is not in frame	Check sequence
No peptide presentation on the cell surface (method: indirect immunofluorescence)	Failure during performing indirect immunofluorescence techniques	Use other primary or fluorescently conjugated secondary antibody
		If issue still exist clone mCherry sequence C-terminally
No peptide presentation on the cell surface (method: direct immunofluorescence)	Failure during transfection	Improve transfection efficiency
	mCherry is not in frame	Check sequence
Control antibody binds on the cells (method: ELISA based)	Antibody concentration too high	Make dilutions of your control antibody
	Cells dried during washing steps → plasma membrane breaks and intracellular epitopes becoming accessible for control antibodies	Ensure that cells never dried while performing ELISA steps

5. References

Background

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