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MEXi

Expression and Purification of Recombinant Proteins from Mammalian Cells using Strep-tag®

Instruction Manual

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This manual can be downloaded under **www.iba-lifesciences.com**

1 Introduction

The use of mammalian cells for the expression of recombinant proteins, particularly of secreted proteins, gains widespread use. Complex glycosylation and accurate protein folding – tasks where *E. coli* or other hosts often fail - make this host highly attractive although culturing and transfection costs are still significant.

Our aim for the development of MEXi (Mammalian Expression IBA) was to provide an economic mammalian expression system by developing a small, well transfecting expression vector backbone (pDSG-IBA) and adapting a HEK293/EBNA cell line to suspension growth in an affordable cell culturing medium. Furthermore, we strived for low biotin content in the cell culturing medium to adapt the expression system to our Strep-tag® protein purification technology providing highly pure recombinant proteins using physiological conditions.
Especially our high affinity 3rd generation Strep-tag system based on the recently developed Strep-Tactin®XT is very well suited for efficient purification of low concentrated proteins from cell culture supernatants.

Moreover, a complete product portfolio beyond protein expression and purification is available at IBA when using Strep-tag® technology, e. g. reagents for specific detection (StrepMAB-Immo, StrepMAB-Classic and Strep-Tactin®(XT) conjugates (enzymatic or fluorescent)) and assay (Strep-Tactin®(XT) coated microplates and Twin-Strep-tag Capture Kit). Thus, the entire research task can be easily performed using synchronized protocols within one technology platform.

2 General procedure of the MEXi system

2.1 Workflow

pDSG-IBA

Combinatorial Sites

Cloning of GOI into pDSG-IBA

↓

Transfection of MEXi 293E cells
in MEXi Transfection Medium

↓

Transient Expression with MEXi 293E cells
in MEXi Transfection and Culture Medium

↓

Purification of GOI (e.g. on immobilized
Strep-Tactin®XT binding a Twin-Strep-tag
fusion protein)

↓

Analysis of GOI
- SDS gel
- Western Blot

Fig. 1: Scheme representing the steps from cloning until validation of the purified protein of interest following the MEXi cloning, expression and purification protocol.

2.2 Cloning of GOI into the pDSG-IBA expression vector

For expression of a gene of interest (GOI) it is recommended to use the pDSG-IBA expression vectors, which are part of the StarGate cloning system. Superfluous elements were eliminated to keep the vector small in size for reaching high transfection efficiency and expression yields.

pDSG-IBA features:

- High-level constitutive expression using the CMV promoter
- ColE1 ori and ampicillin resistance for propagation in *E. coli*
- *oriP* for replication in mammalian cells (the required EBNA-1 gene for replication in mammalian cells is stable expressed by MEXI 293E cells)
- BM40 option for secretion of the protein into the medium
- Different affinity tags (we recommend the Twin-Strep-tag® for secreted proteins)

Features:		**pDSG-IBA vector system**
Replication	***E. coli***	ColE1 ori
	Mammalian cells	*oriP*
	EBNA-1 for *oriP*	no (stable expression in host cell required)
Promoter	**CMV**	yes
Signal peptide	**BM40**	with and without
N-/C-terminal tag	**Strep-tag®II**	yes/yes
	Twin-Strep-tag(R)	yes/yes
	His-tag	yes/yes
	without tag	yes

pDSG-IBA vectors are available as part of the StarGate cloning system but can also be used for direct gene insertion. In latter case, the GOI is amplified by dedicated PCR primers and the PCR product is inserted by a one-tube reaction into the appropriate pDSG-IBA expression vector. After verification of cloning, the resulting expression vector can be directly used for transfection of the MEXi 293E cell line. More information about this cloning procedure and a detailed protocol is available at https://www.iba-lifesciences.com > IBA Manuals > Manual StarGate Direct Transfer Cloning.

The StarGate cloning system also offers the possibility of cloning the GOI into other vector backbones. This is a fall back option if the recommended starting point pDSG-IBA expression system does not work satisfactory for a given GOI and if, therefore, expression conditions need to be optimized. Other mammalian expression vectors within StarGate are derivatives of the pESG- and pCSG-IBA vectors. Check www.iba-lifesciences.com > Mammalia for details.

2.3 Preparation and cultivation of MEXi 293E cells

Thawing MEXi 293E cells from liquid nitrogen

The detailed procedure of thawing cells is described in section 3.4. After thawing, cells will need approximately 1–2 weeks (up to three passages) to fully recover and to reach sufficient viability.

Propagation of MEXi 293E cells

Change the medium regularly and subculture the cells when the cell density reaches 1.5–3.0x10^6 cells/ml. Split cells every 3 to 4 days. High cell viability and a certain cell density are very important for efficient transfection and satisfactory subsequent protein expression levels. MEXi 293E cells should be propagated in the optimized MEXi Culture Medium (MEXi-CM).

2.4 Transfection

In order to obtain the best transfection efficiency MEXi 293E cells need to be transferred from the MEXi Culture Medium into the MEXi Transfection Medium (MEXi-TM).

It is recommended to transfect MEXi 293E cells using linear polyethlenimine (PEI) since PEI is very potent and cost effective. Please note that there exist different PEI formulations from different suppliers. Furthermore, there exist different protocols for solving PEI. This can lead to different optimal PEI concentrations for the transfection than the concentration provided in this manual. Therefore, we recommend a titration (e.g. 4.5, 5.0 and 5.5 mg/l) to determine the PEI concentration providing the most efficient transfection. However, the concentrations provided in this manual should be a good initial point for the experiments.

2.5 Expression

Transient protein expression from pDSG-IBA vectors is driven by the strong, constitutively active CMV promoter.

2–4 hours after transfection of cells in MEXi-TM medium, expression of the protein of interest (POI) is continued by adding the same volume of MEXi-CM. For reaching optimal expression levels and high product purity, expression should be continued until viability of the MEXi 293E cells drops to approx. 75 %. Harvest (in case of cytosolic expression) or remove (in case of secreted proteins) the cells at this point of time. If viability drops below 75 %, product purity and product quality can be negatively affected due to cell lysis and protein degradation.

2.6 Purification

The purification of recombinant proteins via Strep-tag®II or Twin-Strep-tag® is easy, straightforward and user-friendly. In combination with the recently developed high affinity Strep-Tactin®XT the purification of highly diluted proteins from cell culture supernatants becomes more efficient.
The complete purification procedure can be performed under nearly physiological conditions. Only contaminating biotin (in the medium or in the cytosol) has to be respected (see below and 4.1.2).

Important note	**Free biotin in cell culture media or cytosolic biotinylated proteins hamper purification efficiency** Most commercially available cell culture media contain significant amounts of free biotin as a supplement (MEXi-TM is free of biotin and MEXi-CM contains 120 µg/l) while the cell internal content of biotinylated proteins is rather low. Free biotin binds to Strep-Tactin®. Thereby, it prevents binding of the Strep-tag® fusion proteins and, moreover, inactivates Strep-Tactin® resins. Therefore, it has to be removed or masked prior to affinity chromatography. The best and simplest precaution is to add stoichiometric amounts of avidin for irreversible masking of biotin prior to chromatography. We recommend BioLock (Cat. no. 2-0205-050) which is an affordable avidin containing biotin blocking solution. An overview on biotin contents in standard cell culture media or in the cytosol of HEK 293 can be found at http://www.iba-lifesciences.com > Troubleshooting > Biotin Blocking.

3 Required products and preparatory steps

3.1 Products available at IBA

Reagent	Order No.
pDSG-IBA Acceptor Vectors	5-52XX-001
MEXi 293E cell line	2-6001-001
MEXi Culture Medium (MEXi-CM)	2-6010-010
MEXi Transfection Medium (MEXi-TM)	2-6011-010
BioLock	2-0205-050
Strep-Tactin/Strep-Tactin®XT® wash buffer (10x Buffer W)	2-1003-100

3.2 Products from other suppliers

Reagent	Supplier	Order No.
Distilled water e.g. Ampuwa	Fresenius Kabi	
Dulbecco's PBS (1x)	Capricorn	PBS-1A
G-418 Sulphate (50 mg/ml)	Roth	CP11.3
GlutaMAX (100x)	Gibco	35050
25 kDa linear Polyethylenimines (PEI)	Polysciences	23966
Plasmid DNA MiniPrep Kit	Qiagen	27104
Plasmid DNA MaxiPrep Kit	Qiagen	12162

3.3 Preparation of MEXi Culture and Transfection Medium

Preparatory work	Add 1.05 ml G-418 sulphate (stock solution: 50 mg/ml) and 42 ml GlutaMAX (stock solution: 200 mM) to 1 l of MEXi-CM or MEXi-TM media resulting in 50 mg/l G-418 and 8 mM GlutaMAX. Store the ready-to-use medium at 4 °C.

3.4 Seed and recovery of cryopreserved MEXi 293E cells

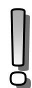
Protocol

1. Fill 20 ml of the prepared MEXi-CM into a 125 ml Erlenmeyer flask, and place the flask for 60 min in an incubator at 37 °C and 5 % CO_2 using gentle shaking to pre-warm the medium and to stabilize pH.
2. Take the MEXi 293E vial out of the cryo container and quickly transfer it to a water bath at 37 °C for thawing. Just before complete thawing, clean the outside of the vial with 70 % ethanol and place it under a clean bench.

continue page 12

Protocol, continued

3. Transfer the cells into a 50 ml centrifugation tube pre-filled with 20 ml of 4 °C cold MEXi-CM.



Note: Avoid any air bubbles during this step, for example during pipetting, as they introduce shear forces and damage the cells.

4. Centrifuge the tube for 5 min at 100 x g, 4 °C and discard the supernatant to remove the DMSO from the storage medium (in which MEXi 293E cells are shipped).
5. Re-suspend the cells with pre-warmed medium of step 1.
6. Transfer the cells into the 125 ml Erlenmeyer flask.

Note: Avoid bubbles and strong shear forces during pipetting.

7. Take an aliquot to determine cell density and viability and incubate the cells at 37 °C and 5 % CO_2 on an orbital shaker platform at 110 rpm-125 rpm (hub 50 mm).

Note: Subculture cells a minimum of three additional passages after thawing before use in transfection experiments to allow recovery from thawing.

3.5 Cultivation of MEXi 293E cells

Protocol

1. Subculture cells every 3 to 4 days (e.g. Monday and Thursday) when cell density reaches 1.5–3.0 x 10^6 cells/ ml by diluting into fresh MEXi-CM. Viability should be > 90 %. MEXi 293E cells can grow to higher densities without loss of viability. However, in some cases cells show lower transfection efficiency when reaching significant higher cell densities than 3.0 x 10^6 cells/ml during cultivation a few days before transfection.
2. Determine cell density and viability using standard methods (e.g. Neubauer chamber, cell counter or flow cytometer etc.).
3. Seed cells in pre-warmed MEXi-CM (37 °C) at a density between 3.0 x 10^5 cells/ml and 5.0 x 10^5 cells/ml. Observe the maximum filling volumes as outlined in the table below.
4. Incubate in humidified air at 37 °C and 5 % CO_2 on an orbital shaker platform at 125 rpm (hub 50 mm).

Important notes

Maximum filling volumes in different shaker flasks

Volume shaker flask	Maximum filling volume
125 ml	30 ml
500 ml	150 ml
2000 ml	500 ml

4 MEXi transfection, expression and purification protocol

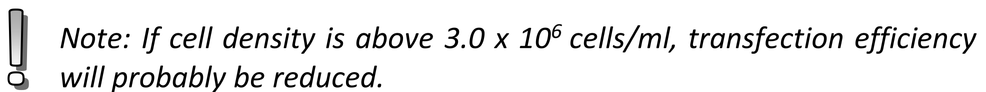
4.1 Protein production in MEXi 293E cells

4.1.1 Transient transfection of MEXi 293E cells with PEI

Protocol

Prior to transfection, MEXi 293E cells need to be transferred from the MEXi Culture Medium into the MEXi Transfection Medium. For efficient transfection cells should be seeded at 1.5 x 10^6 cells/ml.

1. Determine cell density and viability. Viability should be above 90 %. Otherwise: continue culturing cells until viability is above 90 %. (Cells with low viability cannot be well transfected.)

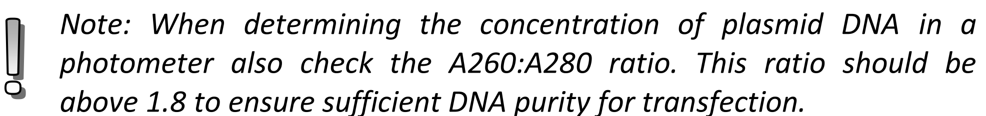


Note: If cell density is above 3.0 x 10^6 cells/ml, transfection efficiency will probably be reduced.

2. Pre-warm MEXi-TM in a culture flask at 37 °C and 5 % CO_2 for 1 h to equilibrate pH in the medium.

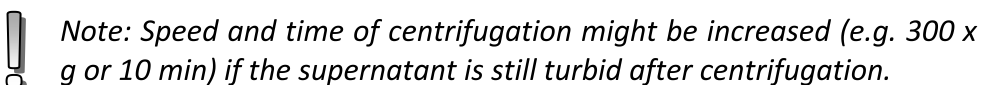
   Thaw the PEI stock solution (1 mg/ml) at 37 °C until the solution is clear. Once the PEI solution is thawed, vortex vigorously for a few seconds.

3. Allow the DNA solution to adjust to room temperature.



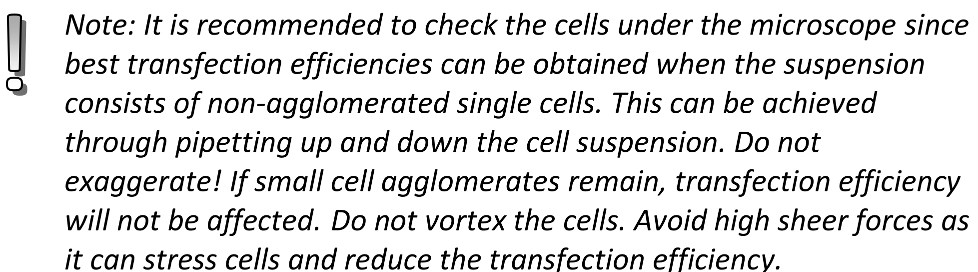
Note: When determining the concentration of plasmid DNA in a photometer also check the A260:A280 ratio. This ratio should be above 1.8 to ensure sufficient DNA purity for transfection.

4. Determine the amount of cell suspension, which is necessary to inoculate the culture flask with 1.5 x 10^6 cells/ml and centrifuge the appropriate amount of cells at 100 x g for 5 min at room temperature (23 °C).



Note: Speed and time of centrifugation might be increased (e.g. 300 x g or 10 min) if the supernatant is still turbid after centrifugation.

5. Discard the supernatant and re-suspend the cells in the appropriate volume of MEXi-TM prepared according to step 2.



Note: It is recommended to check the cells under the microscope since best transfection efficiencies can be obtained when the suspension consists of non-agglomerated single cells. This can be achieved through pipetting up and down the cell suspension. Do not exaggerate! If small cell agglomerates remain, transfection efficiency will not be affected. Do not vortex the cells. Avoid high sheer forces as it can stress cells and reduce the transfection efficiency.

continue page 14

Protocol, continued

6. Inoculate the prepared culture flask (see step 2) to a cell density of 1.5 x 10^6 cells/ml.
7. Add plasmid DNA to a final concentration of 1.5 mg/l directly to the cells and incubate the culture for 10 min on a shaker in order to achieve a homogeneous suspension.
8. Add linear PEI drop by drop to a final concentration of 4.5 mg/l–5.5 mg/l. Shake the flask for a few seconds immediately after adding PEI.

Note: A titration (e.g. 4.5, 5.0 and 5.5 mg/ml) to determine the PEI concentration providing the most efficient transfection might be necessary (see 2.4 Transfection)

9. Shake for 2–4 hours in humidified air at 37 °C and 5 % CO_2 on an orbital shaker platform at 125 rpm (hub 50 mm).
10. Dilute transfected cell culture 1:2 with MEXi-CM (resulting cell density = 0.75 x 10^6 cells/ml) and continue culturing.

Note: A temperature shift to 32 °C shall not be performed prior to cell density has reached 3.0 x 10^6 cells/ml (usually achieved 48 hours post transfection).

11. Check cell viability regularly.
12. Continue cell culturing until viability drops to 75 %.

Note: Because MEXi 293E cells can grow to high densities, large cell clumps may form after a few days. Refer to the protocol in the Appendix 5.2 to break up cell clumps in order to increase the accuracy of your measurements.

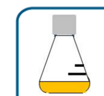
4.1.2 Preparing the cell culture supernatant for (Twin-) *Strep*-tag® affinity purification

Protocol

Important note: We strongly recommend the use of the Twin-Strep-tag® when the recombinant protein is purified from large volumes. Please refer to Schmidt *et al.* (2013), *for further information.*

1. When viability of the transfected culture drops to 75 %, harvest cell culture supernatant. Centrifuge cell suspension at 300 x g for 10 min at 4 °C to harvest cells in case of cytosolic expression or to remove cells from the cell culture supernatant in case of secreted proteins.
2. Add 0.11 volumes 10x Buffer W (e.g. for 1000 ml culture 110ml 10x Buffer W) to the supernatant. Add 1 ml BioLock Biotin Blocking solution to 1 l of supernatant.
3. Incubate for 20 min. Long incubation (e.g. overnight) is possible but will not increase purification efficiency.
4. Centrifuge the supernatant at 10.000 x g for 20 min at 4 C to clear the supernatant and to remove small particles which may clog the column.

continue page 15



5. Check pH and adjust it, if necessary.

Note: For purification Twin-Strep-tag® or Strep-tag®II proteins with Strep-Tactin® the pH should be > pH 7 better pH 8. However, for Strep-Tactin®XT Superflow® and Strep-Tactin®XT 4Flow® the pH can vary from 6-10 and 4-10, respectively.

6. Take a sample of the supernatant for analysis.

4.2 Strep-Tactin®XT purification short protocol

In case of Strep-tag®II or Twin-Strep-tag® as purification tag, continue as follows. If another purification tag is used please refer to the respective purification protocol.

Purification of recombinant protein on a **Strep-Tactin®XT** column:

Cat.no	0.2 ml	1 ml	5 ml	10ml
Strep-Tactin®XT Superflow®	2-4011-005	2-4012-001	2-4013-001	2-4014-001
Strep-Tactin®XT Superflow® high capacity	2-4031-005	2-4032-001	2-4033-001	2-4034-001
Strep-Tactin®XT 4Flow®	2-5011-005	2-5012-001	2-5013-001	2-5014-001
Strep-Tactin®XT 4Flow® high capacity	2-5031-005	2-5032-001	2-5033-001	2-5034-001

Important notes

Generally, it is recommended to perform chromatography at 4 °C. Depending on the individual equipment this is not always possible and chromatography has to be performed at room temperature. If columns are stored at 4°C and transferred to room temperature air bubbles may form due to restricted solubility of air at elevated temperatures. Therefore, it is recommended to equilibrate the columns immediately after exposure to higher temperatures with buffer which is adjusted to room temperature.

Protocol

1. Remove storage buffer of the column.
2. Equilibrate the Strep-Tactin®XT column with 2 column volumes (CVs) Buffer W. The column cannot run dry under gravity flow.

   Note: Use buffer without EDTA for metalloproteins.
3. Load the supernatant on the column. For application of larger volumes the use of WET FRED might facilitate the procedure.
4. Wash the column 5 times with 1 CV 1x Buffer W, after the supernatant has completely entered the column. Collect the flow through in fractions with a size of 1 CV.
5. Add 6 times 0.5 CV of 1x Buffer BXT for elution and collect each

fraction. The major protein should elute in fractions 2, 3 and 4.

Option: To get high protein concentrations in one fraction add 0.6 CV as elution fraction 1 (E1), then 1.6 CV (E2) and finally 0.8 CV (E3). Main protein content should be in E2.

The detailed purification protocol for (Twin-) Strep-tag® fusion proteins can be downloaded from http://www.iba-lifesciences.com/download-area.html > IBA Manuals (Download):

- Strep-TactinXT_Purification_Manual.pdf
- Strep-TactinXT_Purification_Short_Protocol.pdf

IBA offers optional products:

WET-FRED
This applicator is suitable for either Strep-Tactin® 1 ml or 5ml and 10ml gravity flow columns. WET FRED enables application of large cell culture supernatant volumes to a gravity flow column conveniently and in a simple way.
Cat.no: 1 ml: 2-0911-001 and 5 ml: 2-0910-001

Cartridges for Äkta usage:
IBA also provides 1 ml and 5 ml Strep-Tactin®XT cartridges for Äkta users.

Cat.no	1 ml	5 ml
Strep-Tactin®XT Superflow®	2-4021-001	2-4022-001
Strep-Tactin®XT Superflow® high capacity	2-4025-001	2-4026-001
Strep-Tactin®XT 4Flow®	2-5021-001	2-5022-001
Strep-Tactin®XT 4Flow® high capacity	2-5025-001	2-5026-001

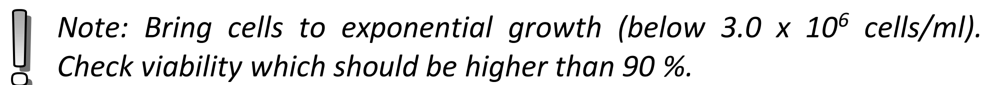
MagStrep “type3” XT beads
Use for small scale purification e.g. to screen for optimal expression conditions.
The Strep-Tactin®XT coated magnetic beads have a high binding capacity combined with very low non-specific protein binding. Elution can therefore also be performed by boiling in denaturing SDS gel loading buffer instead of using biotin if the isolated protein does not need to be functional.
Cat.no: 2-4090-002

More information: **www.iba-lifesciences.com.**

5 Appendix

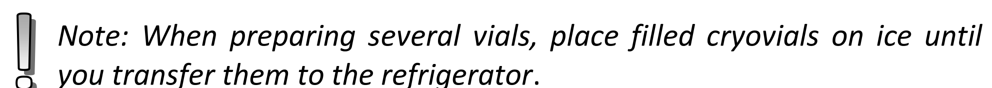
5.1 Recommended Cryopreservation of MEXi 293E cells

Protocol



Note: Bring cells to exponential growth (below 3.0 x 10^6 cells/ml). Check viability which should be higher than 90 %.

1. Prepare a sufficient amount of cryovials and place them on ice until use.
2. Immediately before use, mix 0.9 ml of MEXi-CM with 0.1 ml of DMSO for every ml freezing medium.
3. Filter-sterilize the freezing medium and pre-refrigerate it at 4 °C or place it on ice.
4. If using “Mr. Frosty” for the freezing procedure, pre-cool it at 4 °C.
5. Determine the cell density.
6. Calculate the volume of cell suspension which is necessary to freeze 1.1 x 10^7 cells per cryovial.
7. Mix the culture by shaking and pipette the calculated amount of cell suspension into a 15 ml or 50 ml centrifugation tube with conical bottom.
8. Centrifuge at 100 x g for 5 min at room temperature.
9. Discard the supernatant. The cell pellet must remain in the tube.
10. Re-suspend cells in the freezing medium to achieve a cell density of 7.33 x 10^6 cells/ml and add 1.5 ml of the suspension into a 2 ml cryovial.
11. Incubate the cryovials for 15 min at 2–8 °C to allow the DMSO to penetrate into the cells.



Note: When preparing several vials, place filled cryovials on ice until you transfer them to the refrigerator.

12. Transfer the vials into “Mr. Frosty” and place it in a -80 °C freezer for 24 h.
13. Remove the vials from -80 °C freezer and transfer them into a cryo storage system at -140 °C to -196 °C (in liquid nitrogen).

5.2 Preparation of cells for measuring viability and cell density

Protocol

During expression of the GOI MEXi 293E cells can grow to high cell densities and form big cell clumps. These cell clumps affect analysis like measurement of cell density and viability. Use the following procedure to break up cell clumps.

If cells do not grow to very high densities (< 4 x 10^6 cells/ml), vigorous vortexing (20 – 70 seconds) might be sufficient to break up cell clumps.

1. Place the cell culture flask under a clean bench.
2. Transfer 200 µl of cell suspension into a 1.5 ml reaction tube and centrifuge at 100 x g for 3 min at room temperature.
3. Remove carefully 180 µl of supernatant without disturbing the pellet and discard the supernatant.
4. Add 180 µl of Dulbecco’s PBS with 5 mM EDTA and 1 % BSA, re-suspend the pellet and centrifuge at 100 x g for 3 min at room temperature.
5. Remove carefully 180 µl of supernatant and discard it.
6. Add 180 µl of TrypLE™ (Gibco, cat no. 12604013), re-suspend the pellet and incubate for 2 min at room temperature.
7. Mix the sample and centrifuge at 100 x g for 1 min 30 s at room temperature.
8. Remove carefully 180 µl of supernatant and discard it.
9. Add 180 µl Dulbecco’s PBS with 5 mM EDTA and 1 % BSA and re-suspend the pellet by pipetting several times.
10. Vortex the sample vigorously for 30 s–90 s and proceed with your measurement.

6 Troubleshooting

Expression		
Problem	**Cause**	**Comments and suggestions**
Low expression levels or no expression	Sequence error, mutation	Verify sequence and reading frame
	Low transfection efficiency	Make sure that the cells are transferred in MEXi Transfection Medium. Optimize DNA and PEI concentration (recommended: 1.5 mg/l DNA; 4.5–5.5 mg/l PEI).
	Protein is toxic	Some proteins are inhibiting cell growth or induce apoptosis. In some cases signaling-inactive forms can be expressed at high levels.
	Cell viability (fitness)	If cells are in a high passage or cell growth or viability is low transfection might be inefficient. Use cells which are in logarithmic growth phase (below 3 x 10^6 cell/ml) and show a high viability (e.g. > 90 %).
	Media conditions	For expression use MEXi-TM and MEXi-CM in a 1:1 ratio.
Protein is not secreted	Signal sequence present	Remove all signal sequences from the coding region.

7 References

Schmidt & Skerra (2015) The Strep-tag System for One-Step Affinity Purification of Proteins from Mammalian Cell Culture. *Methods in Molecular Biology,* Vol. 1286, DOI 10.1007/978-1-4939-2447-9_8

Schmidt *et al.*, (2013) Development of the Twin-Strep-tag® and its application for purification of recombinant proteins from cell culture supernatants. *Protein Expr Purif*. Nov;92(1):54-61. doi: 10.1016/j.pep.2013.08.021

Schmidt & Skerra (2007) The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins *Nat Protoc.* 2(6):1528-35

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