Baculovirus-driven protein expression in insect cells: A benchmarking study

Peggy Stolt-Bergner, Christian Benda, Tim Bergbrede, Hüseyin Besir, Patrick H.N. Celie, Cindy Chang, David Drechsel, Ariane Fischer, Arie Geerlof, Barbara Giabbai, Joop van den Heuvel, Georg Huber, Wolfgang Knecht, Anita Lehner, Regis Lemaitre, Kristina Nordén, Gwynn Pardee, Ines Racke, Kim Remans, Astrid Sander, Judith Scholz, Magda Stadnik, Paola Storici, Daniel Weinbruch, Isabel Zaror, Linda H.L. Lua, Sabine Suppmann

PII: S1047-8477(18)30066-2
DOI: https://doi.org/10.1016/j.jsb.2018.03.004
Reference: YJSBI 7171

To appear in: *Journal of Structural Biology*

Received Date: 7 December 2017
Revised Date: 27 February 2018
Accepted Date: 10 March 2018

Please cite this article as: Stolt-Bergner, P., Benda, C., Bergbrede, T., Besir, H., Celie, P.H.N., Chang, C., Drechsel, D., Fischer, A., Geerlof, A., Giabbai, B., van den Heuvel, J., Huber, G., Knecht, W., Lehner, A., Lemaitre, R., Nordén, K., Pardee, G., Racke, I., Remans, K., Sander, A., Scholz, J., Stadnik, M., Storici, P., Weinbruch, D., Zaror, I., Lua, L.H.L., Suppmann, S., Baculovirus-driven protein expression in insect cells: A benchmarking study, *Journal of Structural Biology* (2018), doi: https://doi.org/10.1016/j.jsb.2018.03.004

Baculovirus-driven protein expression in insect cells: A benchmarking study

Peggy Stolt-Bergner[a], Christian Benda[b], Tim Bergbrede[c], Hüseyin Besir[d], Patrick H. N. Celie[e], Cindy Chang[f], David Drechsel[g], Ariane Fischer[b], Arie Geerlof[h] , Barbara Giabbai[i], Joop van den Heuvel[j], Georg Huber[h], Wolfgang Knecht[k], Anita Lehner[a], Regis Lemaitre[g], Kristina Nordén[k], Gwynn Pardee[l], Ines Racke[d], Kim Remans[d], Astrid Sander[m], Judith Scholz[n], Magda Stadnik[e], Paola Storici[i], Daniel Weinbruch[o], Isabel Zaror[l], Linda H. L. Lua[f*], Sabine Suppmann[n*]

[a] Vienna Biocenter Core Facilities GmbH, Dr. Bohr-gasse 3, 1030 Vienna, Austria

[b] Max-Planck Institute of Biochemistry, Structural Cell Biology Department, Am Klopferspitz 18, 82152 Martinsried, Germany

[c] Lead Discovery Center GmbH, Otto-Hahn-Str. 15, 44227 Dortmund, Germany

[d] European Molecular Biology Laboratory (EMBL) Heidelberg, Protein Expression and Purification Core Facility, 69117 Heidelberg, Germany

[e] NKI Protein Facility, Division of Biochemistry, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

[f] Protein Expression Facility, The University of Queensland, Brisbane, Queensland 4072, Australia

[g] Protein Expression Purification and Characterization facility, Max Planck Institute of Molecular Molecular Cell Biology and Genetics, Pfotenhauerstr. 108, D-01307 Dresden, Germany

[h] Helmholtz Zentrum München, Protein Expression and Purification Facility, Institute of Structural Biology, Ingolstädter Landstraße 1, D-85764 Neuherberg

[i] Structural Biology Laboratory, Elettra-Sincrotrone Trieste S.C.p.A., Trieste 34149, Italy

[j] Helmholtz Centre for Infection Research, Department Structure and Function of Proteins Inhoffenstrasse 7, 38124 Braunschweig, Germany

[k] Lund University, Lund Protein Production Platform (LP3), Sölvegatan 35, SE-223 62, Lund, Sweden

[l] Novartis Institutes for BioMedical Research, Emeryville, California, US

[m] Max Planck Institute of molecular physiology, Dortmund Protein Facility, Otto-Hahn-Str. 11, 44227 Dortmund, Germany

[n]Max-Planck Institute of Biochemistry, Biochemistry Core Facility, Am Klopferspitz 18, 82152 Martinsried, Germany

[o]Coriolis Pharma, Biopharmaceutical Research and Development Service, Am Klopferspitz 19,82152 Martinsried, Germany

* co-corresponding authors E mail l.lua@uq.edu.au; suppmann@biochem.mpg.de

Abstract

Baculovirus-insect cell expression system has become one of the most widely used eukaryotic expression systems for heterologous protein production in many laboratories. The availability of robust insect cell lines, serum-free media, a range of vectors and commercially-packaged kits have supported the demand for maximizing the exploitation of the baculovirus-insect cell expression system. Naturally, this resulted in varied strategies adopted by different laboratories to optimize protein production. Most laboratories have preference in using either the *E. coli* transposition-based recombination bacmid technology (e.g. Bac-to-Bac®) or homologous recombination transfection within insect cells (e.g. flashBAC™). Limited data is presented in the literature to benchmark the protocols used for these baculovirus vectors to facilitate the selection of a system for optimal production of target proteins. Taking advantage of the Protein Production and Purification Partnership in Europe (P4EU) scientific network, a benchmarking initiative was designed to compare the diverse protocols established in thirteen individual laboratories. This benchmarking initiative compared the expression of four selected intracellular proteins (mouse Dicer-2, 204 kDa; human ABL1 wildtype, 126 kDa; human FMRP, 68 kDa; viral vNS1-H1, 76 kDa). Here, we present the expression and purification results on these proteins and highlight the significant differences in expression yields obtained using different commercially-packaged baculovirus vectors. The highest expression level for difficult-to-express intracellular protein candidates were observed with the EmBacY baculovirus vector system.

Key words

Baculovirus-insect cell system; benchmark

Abbreviations: AcMNPV, *Autographa californica* nuclearpolyhedrovirus; BAC, bacterial artificial chromosome; BEVS, baculovirus expression vector system; DNA, deoxyribonucleic acid; ds, double stranded; E. coli, *Escherichia coli*; YFP, yellow fluorescent protein; kDa, kilo Dalton; MOI

multiplicity of infection; p10, p10 baculoviral late promoter; polh, polyhedrin baculoviral very late promoter; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IMAC, immobilized metal affinity chromatography; Sf21, *Spodoptera frugiperda* cell line 21; Hi5, *Trichoplusia ni* cell line; P4EU; Protein Production and Purification Partnership in Europe

1. Introduction

Baculovirus-insect cell expression system is a workhorse in many research laboratories for recombinant protein production. Its superiority for the expression of complex proteins to prokaryotic *Escherichia coli* expression host is well-demonstrated by the use of the technology for routine production of glycoproteins, G-protein-coupled receptors (GPCRs), virus-like particles (VLPs) and 'difficult-to-express' mammalian proteins. Further validation of the baculovirus expression technology is demonstrated by the commercial manufacture of human and veterinary vaccines using this expression system, including GSK's human papillomavirus VLP vaccine (Deschuyteneer et al., 2010), Protein Sciences' influenza hemagglutinin (HA) vaccine (Cox and Hollister, 2009) and porcine circovirus ORF2 vaccine (Fan et al., 2007). A steady increase in both published scientific papers and patents citing the use of baculovirus-insect cell expression system is observed over the last 30 years (van Oers et al., 2015).

Although baculovirus-insect cell expression system is a ready-to-use system, largely due to the availability of commercial baculovirus expression kits complete with vector, cell line, medium and detailed protocols, there are multiple factors to consider and optimize for the production of proteins. Developments in engineering baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) for improved recombination efficiency (bacmid technology or homologous recombination), ease of production for multisubunit protein complexes (single baculovirus vector) and improved glycoprotein expression (gene deletion or inclusion) further broaden the variety of baculovirus expression systems. The two most commonly used commercial baculovirus systems are Bac-to-Bac® (transposition-based bacmid technology) and *flash*BAC™ (homologous recombination). In general, a research laboratory chooses one system over another depending on the cost and availability, whereas little attention is given to the protein quality and quantity of the same protein construct across different baculovirus expression systems. Similarly, there is limited literature to guide the selection of the optimal insect cell line or the inherent cell culture medium. Clonal isolates of *Spodoptera frugiperda* and *Trichoplusia ni* are the most commonly used cell lines for protein production.

The network of P4EU (Protein Production and Purification Partnership in Europe) has international protein production facilities spread across > 40 countries. These facilities' core service is to accelerate research through the provision of high quality proteins to researchers working in diverse disciplines. As an effort to enhance shared knowledge and learnings in the field of recombinant protein production, a benchmarking was conducted to compare the efficiency and productivity of baculovirus-insect cell expression methods adopted in the individual laboratories. This initiative examined and compared the performance of each participating laboratory on the production of four selected intracellular protein candidates using its existing materials and processes. This benchmarking highlights the range of variables between laboratories. Here, we present the results obtained by thirteen laboratories on the four intracellular protein candidates, narrowing down the single most impactful factor on protein yield when using the baculovirus-insect cell expression system.

2. Materials and methods

2.1. Selected target genes

Four genes were selected for benchmarking (Table 1): mouse Dicer-2 (Dcr-2), MW 204 kDa; human ABL1 wildtype, MW 126 kDa; human FMRP, MW 68 kDa; viral vNS1-H1, MW 76 kDa. All proteins localized intracellularly and were N-terminally fused to 6xHis to enable a uniform sample analysis and protein purification procedure. The selection of these candidates was to challenge the participating laboratories with difficult-to-express proteins. Expression of these proteins in *E.coli* had previously failed. When previously expressed in insect cells using BEVs, low yields (< 1 mg/L) and poor protein stability or solubility were obtained. *Drosophila melanogaster* Dicer-2 is a member of the ribonuclease III family and is essential in the host defense against RNA viruses mediated by the RNA induced silencing complex (RISC). The role of Dicer-2 is to trim double-stranded RNAs into small-interfering RNAs (siRNAs), which play an essential role in RNA interference (Lee et al., 2004). ABL1 is a non-receptor tyrosine-protein kinase that plays a role in many key processes linked to cell growth

and survival such as cytoskeleton remodelling in response to extracellular stimuli, cell motility and adhesion, receptor endocytosis, autophagy, DNA damage response and apoptosis (Colicelli, 2010). ABL1 and Dicer-2 protein samples purified from insect cells using BEVs were heavily degraded. Loss of Fragile X mental retardation protein (FMRP) causes Fragile X syndrome which is the most common genetically inherited form of cognitive impairment (Till, 2010). The isoform 2 of FMRP used in this study was expressed at 1.5 mg/L but was only partly soluble. NS1-H1 is a major viral nonstructural protein [H-1 parvovirus] of unknown function.

2.2. Design of the study

The selected target genes listed in Table 1, originally provided in pFastBac backbones were re-cloned into pBac1 for users of *flash*BAC™ and similar baculovirus variants. The design of the benchmarking study is shown in Fig. 1. The pFastBac and pBac1 plasmids were distributed to the respective BEVS users, among them seven laboratories using Bac-to-Bac®, MultiBac® or EMBacY and seven laboratories using *flash*BAC™ and similar baculovirus variants. Participating laboratories were asked to proceed with their routine procedures for virus generation and small-scale optimization; to produce each construct at 500 mL scale; to sample triplicates of 500 µL cell pellets for total lysate analysis and duplicates of 10 mL cell pellets for protein purification. Cell pellets were washed twice in PBS before freezing in liquid nitrogen. Non-infected control cells were included for each cell type used, cell counts and viability recorded and provided together with protocols (Table 2 and Supplementary S1). The 500 µL cell pellets were shipped on dry ice to two different sites (VBCF Vienna and MPIB Martinsried) for central sample analysis by two different and independent methods to exclude any method bias. 10 mL frozen cell pellets that were prepared for protein purification and quality assessment were also centralized at one site (MPIB Martinsried) to exclude impact of protein purification on the benchmark results.

2.3. Participating Protein Production Facilities

This benchmark study was initiated by members of the Protein Production and Purification network P4EU (https://p4eu.org) who provide central scientific services for recombinant protein production and purification, mainly in *E. coli*, mammalian and insect cells. Although originally founded as a European network, members also include laboratories from outside the Eurozone. All participants as displayed in the authors list provide services for scientific groups and are typically financially supported by their respective institutions. In order not to compromise participants for potential low performance, especially with respect to the challenging target genes selected for this study, participating labs were anonymized by letters.

2.4. General protein expression procedures

The most relevant parameters for baculovirus-driven protein expression in insect cells adopted by the participating labs are listed in Table 2 (full details described in Supplementary S1). The two different strategies for target gene integration into the baculovirus genome are each represented by seven participating laboratories. Lab E, F, I, H, L, M, and X belong to the group which uses Tn7 transposition-based integration of the target gene from the pFastBac transfer plasmids into the baculovirus genome within *E.coli* cells. Bacmids belonging to this group used in the present study are Bac-to-Bac® (Invitrogen), MultiBac® (Berger et al., 2013) and EMBacY (Bieniossek et al., 2008). With the second strategy, herein called recombination-based transfection, recombinant baculovirus is generated directly in insect cells by cotransfecting the pBac1 transfer plasmids with bacmid DNA. Non-recombinant viral DNA is replication-incompetent, allowing propagation of recombinant virus only. Baculovirus variants used in this study based on this principle are *flash*BAC™ and *flash*BACULTRA™ (Oxford Expression Technologies OET). ProGreen™ (AB Vector) and an in-house *chiA* and *v-cath* deleted bacmid variant "DefBac" (manuscript in preparation by lab U). ProGreen™ and DefBac are recombination-based methods that are adapted from the

*flash*BAC procedure. Lab C, D, F, K, R, U and V belong to the users of recombinant-based transfection. Lab F contributed expression samples for both systems, except NS1-H1 in Bac-to-Bac® and ABL1 in *flash*BACULTRA™. Insect cell lines that have been used by the laboratories are *Spodoptera frugi*perda (Sf21 and Sf9) and / or *Trichoplusia ni* (Hi5). All groups cultivated cells in suspension in different commercial media (except one proprietary recipe) as listed: ESF 921™ or III serum free medium (SFM) (Invitrogen), ESF 921™ (Expression Systems), Insect-XPRESS™ (BioWhittaker), EX-CELL® 405 for Hi5 cells and EX-CELL® 420 for Sf21/Sf9 cells (Sigma), and SFX-Insect (GE Healthcare). The amount of baculovirus used for infecting expression cultures, cell density at infection, expression time and expression temperature was either constant or have been adjusted individually for each of the four constructs (indicated by ranges e.g. virus dilution 1:100 – 1:35).

2.5. Analysis and quantification of protein expression levels

Quantitative comparison of expression levels in total lysates was performed on Amersham Easy SDS-PAGE (MPIB Martinsried) and Simple Western (VBCF Vienna) to cover sample analysis with methods that are complementary in sensitivity, specificity and dynamic range and use different electrophoretic separation principles. Easy SDS-PAGE (Amersham WB system, GE Healthcare) uses conventional acrylamide electrophoresis combined with labelling of lysine residues with sulfonated Cy5 at low dye to protein ratios (Bjerneld et al., 2015). Gels are automatically scanned post-run and quantitative data displayed as peak lists. Sensitivity and dynamic area are high, ranging from 1 ng/µL up to 20 µg/µL. Cell pellets were thawed on ice and resuspended to a concentration of 1 x 10^6 cells/mL in 20 mM Tris pH 8.0 + 0.25% SDS according to the viable cell count, boiled for 5 min and spun down. Gel samples were prepared by mixing 2 µL cell suspension with 17 µL labelling buffer and 1 µL Cy5, incubated 30 min at room temperature, mixed with 20 µL loading buffer, boiled for 3 min and loaded on 13.5% Easy SDS-gels. Data were displayed as % of total peak intensity at

the respective size compared to non-infected control cells, circumventing the need for any normalization. Although being very sensitive, the lack of specificity allows detection of significant overexpression in total lysates versus control cells with a threshold of at least 2.5 % of total. Simple Western (Peggy^{TM}, Protein Simple) is a capillary-based immunoassay platform that performs automated protein separation, detection and quantification. The Peggy^{TM} instrument has similar sensitivity and somewhat improved dynamic range as compared with traditional Western blotting with chemiluminescence, with additional advantages of improved reproducibility due to automation of many manual steps, enabling quantitative comparison of protein amounts across samples (Rustandi et al., 2012). In some cases, proteins may show an aberrant migration behaviour in capillary protein separation. Cell pellets were thawed on ice and resuspended to a concentration of 1 x 10^6 cells / mL in PBS + 0.1% SDS according to the viable cell count. This stock was diluted 4-fold with PBS + 0.1% SDS, and total protein content was measured with the OPA assay (ThermoFisher Scientific) and compared to a standard curve using known concentrations of BSA. If necessary, lysates were further diluted to a concentration below 1 mg/mL to be within the linear range of the OPA assay. The 4-fold diluted total lysate sample was mixed with Simple Western loading buffer (7.5 µL sample and 2.5 µL 4X loading buffer), boiled for 5 min, spun down and 5 µL were loaded into a 384-well Simple Western plate for analysis. For immunodetection, primary anti-penta-His antibody (Qiagen) at a dilution of 1:20 and secondary HRP-labeled anti-mouse antibody (Protein Simple) were used. Peak areas were calculated for each sample using the Compass software (Protein Simple) and normalized using the total protein concentration determined by the OPA assay. Samples outside the linear range of detection (as determined by either no signal or detection of signal saturation, i.e. “burn-out”, using the Compass software) were re-measured using higher or lower dilutions. For conventional SDS-PAGE analysis (Fig. 6), protein samples were analyzed on a 4-12% Bis-Tris SDS-polyacrylamide gel under reduced and denatured conditions. Equal volumes of cell pellets were loaded and

stained with SimplyBlue™ SafeStain (Thermo Fisher Scientific). Target protein expression level as a % of total cellular protein was quantified using Chemi-Doc™ XRS+ imaging system (Bio-Rad Laboratories, CA, USA).

2.6. Protein purification and quality assessment

Prior to processing all benchmark samples, purification of the four 6xHis-fusion proteins by immobilized metal affinity chromatography (IMAC) was optimized with regard to detergents to increase solubility especially of FMRP and with regard to the type of Ni^{2+}-beads optimal for binding large proteins as Dicer-2 and cABL1. Based on these test purifications, different procedures were used for isolation of the four proteins. Buffers contained 50 mM Tris pH 8.0, 500 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, 1 mM TCEP plus imidazole at 10 mM (lysis), 20 mM (wash) and 250 mM (elution) final concentration. Protease inhibitors (final concentration: AEBSF-HCl 1 mM, Aprotinin 2 µg/mL, Leupeptin 1 µg/mL, Pepstatin 1 µg/mL) and 750 U Benzonase (Novagen, Cat No 70664-3) were added to all lysis buffers. For purification of FMRP, Chaps was added to all buffers at 0.5%. Purification of Dicer-2 and ABL1 was automated on a Tecan Freedom Evo 150. Cell pellets from 10 mL cultures were resuspended in 2 mL lysis buffer and lysed by sonication on a Vibra Cell VC750 (Sonics and Materials, Inc, Newton) equipped with a 24 tip horn (Qsonica, LLC, Newton). Lysates were centrifuged for 30 min at 13,200 rpm at 4 °C. 1.3 mL of lysate supernatants were loaded on MediaScout RoboColumns columns (Atoll, Germany) filled with 50 µL Chelating Sepharose FF (GE Healthcare), washed 9x with 800 µL lysis buffer and eluted 3x in 60 µL elution buffer. Cell pellets of FMRP and vNS1-H1 were resuspended in 1.4 mL lysis buffer, lysed in a Dounce homogenizer and centrifuged for 30 min at 20,000 rpm at 4 °C. Lysate supernatants were loaded on 50 µL MagneHis particles (Promega), rotated for 60 min at 4 °C, washed 3x in 200 µL wash buffer and eluted 3x in 60 µL elution buffer. Protein quantitation was performed by Bradford staining (Pierce, Thermo Scientific) and protein containing fractions

were pooled. Sample analysis by Easy SDS-PAGE was performed immediately after elution. For electrophoresis, 2 µL imidazole eluate was mixed with 17 µL labelling buffer and 1 µL Cy5, incubated 30 min at room temperature, mixed with 20 µL loading buffer, boiled for 3 min and loaded on 13.5% Easy SDS-gels. For dynamic light scattering (DLS), 120 µL samples from both purification procedures were transferred into a flat-bottom polystyrene 96 well plate (Greiner, Germany) and analyzed on the Wyatt DynaPro Plate Reader. DLS data were recorded using a small number of short acquisitions (10 acquisitions for 5 sec each) in two consecutive runs. Further replicates were not recorded for reasons of limited protein stability.

3. Results and Discussion

3.1. Output of different facilities: protein expression levels

In a first series of experiments, overexpression levels of all four constructs were analysed by Easy SDS-PAGE and Simple Western. Total lysates were produced on site for the two different measurements (see Materials and Methods) from 500 µL cell pellet aliquots derived from 500 mL-scale productions, that had been performed using the individual routine protocols of the different laboratories (Table 2 and Supplementary S1). Datasets from Easy SDS-PAGE and Simple Western are available for all four constructs and all participants. Representative images for results from Labs L, M and R analysed by Easy SDS-PAGE and Simple Western are shown in Fig. 2A and B. Intensity quantification of Cy5-labelled overexpressed proteins in Easy SDS-PAGE allows for display of expression levels directly as [%] of total peak intensity. Accordingly, Dicer-2 is expressed at levels ranging from 13% - 23%; ABL1 from undetectable to 23%; FMRP from undetectable to 27% and NS1-H1 from undetectable to 16% in Lab M, L and R. It is important to note that for determining expression levels, only proteins of the expected size (‘intact proteins’) were included in

positive results. Fig. 2A illustrates such an example: Dicer-2 is well expressed in lab L, but migrates at 117 kDa according to sizing based on internal molecular weight marker calibration by the Amersham WB software (red arrowhead in Fig. 2A). Such results were considered as 0 % expression level. This degradation product was also detected in the respective Simple Western analysis (Fig. 2B, red arrowhead). The quantitative comparison of expression levels between the 13 different laboratories for all four constructs and the two measurement series are shown in Fig. 3A-D. In order to compare the different datasets side-by-side, the highest value within each dataset was set to 100% performance and all values within the respective dataset normalized in relation to the highest value (original data listed in Supplementary S2). Datasets produced with either Easy SDS-PAGE or Simple Western are in reasonable agreement with one major exception: expression of full-length Dicer-2 by Lab I could not be detected in Simple Western but in Easy SDS-PAGE. As discussed in Materials and Methods, discrepancies may arise from the need to normalize the data to account for differences in total protein levels in the Simple Western analysis and the difference between the detection methods used. Dicer-2 and ABL1 expression by Lab X was not monitored by Simple Western analysis, see * in bar graph (Fig. 3). Most important, all datasets support the following key observations. First, differences in performance between the 13 laboratories are enormous, showing the following range based on Cy5-labelling derived expression levels underlying Fig. 3 A-D : Dicer-2 expression levels range from undetectable (lab C, D, V) to 23 % of total cellular protein (lab M, Figs. 2A and 3A); ABL1 expression levels range from undetectable (lab F, I, L, K, C, D, V) to 23% of total cellular protein (Lab M (Figs 2A and 3B); FMRP is expressed at considerable levels in most labs, with up to 43% of total cellular protein in lab X (Fig. 3C and S2) and NS1-H1 expression levels range from undetectable (lab L, V) to 16% of total cellular protein (lab R, Figs. 2A and 3D). Second, differences in performance are most pronounced for expression of Dicer-2 and ABL1 (Fig. 3AB). 6 out of 13 labs failed to express Dicer-2 at detectable or significant levels and 7 out of 14 labs

expressed ABL1 at levels not detectable by Simple Western analysis. As mentioned in Materials and Methods and also supported by our data, these two proteins have been included in this study to challenge the performance of the individual procedures because they are known to be difficult-to-express and prone to degradation. With regard to these two target proteins, the excellent performance of the procedures used in Lab M, H, X and R are particularly noticeable.

3.2. Output of different facilities: protein yields

Although the analysis of expression levels in total lysates was very informative with regard to differences in performances of the diverse procedures, it does not address their impact on protein solubility and stability. To address these questions, all four proteins were purified as described in Materials and Methods from cell pellets of 10 mL from the previously described 500 mL productions used for analysis of total lysates. Representative Easy SDS-PAGE results for IMAC purifications from Lab M, R and U are shown in Fig. 4A. Intensity quantification of Cy5-labelled purified proteins allows for the display of purity directly as [%] of total peak intensity. As expected, eluates of Dicer-2 and ABL1, both degradation-prone proteins, showed poor purity, less than 10% and 25% respectively. On the other hand, FMRP and NS1-H1 were eluted with purities of up to 66% (lab M) and 96% (lab U) respectively from this single IMAC step. In order to compare performance of all participating labs taking into account the different degrees of purity, the amount of protein eluted from IMAC was corrected by its purity determined by Easy SDS-PAGE (original data listed in Supplementary S3). Again, highest purity-corrected protein yield was set to 100% and all other data related to it (Fig. 4AB). Absolute values for the highest purity-corrected yields obtained were: Dicer-2: 3 mg/L; ABL1: 4 mg/L, FMRP: 25 mg/L; NS1-H1: 18 mg/L. Differences in performance derived from protein yields are in good agreement with results from the analysis of expression levels in total lysates. With regard to Dicer-2 and ABL1, lab M, H, X and R belong to the best

performers, lab E, I and U show medium performance, whereas labs $F_{Bac\text{-}to\text{-}Bac}$, L, K, C, D and V show lowest performance (Fig. 3AB, Fig. 4B). FMRP, also in agreement with expression levels from total lysates was purified at medium to high performance from most labs (Lab M, H, X, I, $F_{Bac\text{-}to\text{-}Bac,}$ E, L, R, U, $F_{flashBAC}$ and K) but at low levels in Lab C, D and V. Most striking for this particular protein is the fact that pronounced differences in expression levels are not necessarily reflected in protein yields. As an example, lab X and lab U showed 43% and 9% expression level in total lysates respectively, but comparable yields from protein purification (Fig. 3C and Fig. 4B). This is probably due to limited solubility of FMRP protein that could not be overcome by adding 0.5% Chaps to all purification buffers (see Methods). For NS1-H1, in analogy to expression levels in total lysates, protein yields are highest in lab M, X, R, U, H, medium in Lab E and low in lab I, L, K, C, D and V (Fig. 3D and Fig. 4B). In order to compare protein homogeneity, dynamic light scattering was recorded for all purified proteins to quantify aggregate content. While purities of Dicer-2 and ABL1 preparations were too low for meaningful data interpretation (data not shown), no significant differences in protein qualities of FMRP and NS1-H1 with respect to aggregate content could be detected (Supplementary S4).

3.3. Trends in performance differences

As described under sections 3.1. and 3.2. and illustrated in Figs. 2 to 4 and Table 2, we have observed major differences in the competence to express the target proteins Dicer-2, ABL1, FMRP and NS1-H1 in insect cells in the participating laboratories. For all constructs, there are a few high performers (M, H, X and R), some medium performers (E, F_{flash}BacULTRA™, K, U) and some very low performers (L, C, D, V). The key question remains, which of the parameters in the respective procedures (Table 2) account(s) for high performance. A specific look at the high performer labs M, H, X and R shows that there is no straightforward answer, while they used 3 different baculovirus vectors; 2 types of cell lines; 4 different media; 3

different flask types; with various amounts of virus used for infecting 500 ml culture (from 1:18 to 1:125); expression times ranging from 48 to 120 h and incubation temperatures at 21 or 27 °C. There are, however, striking relations. First of all, in direct comparison of the performance of the baculoviral vectors by plotting the scored overall performance of expression levels and protein yields of laboratories using non-modified baculoviruses (Bac-to-Bac® and ProGreen™) versus all laboratories using baculoviruses carrying gene deletions Δ*chiA*, Δ*v-cath*/*chiA* or Δ*v-cath/chiA/p10/p74/p26* (MultiBac®, EMBacY, *flash*Bac™ *flash*BacULTRA™, DefBac) indicates a clear benefit of the *v-cath* and *chiA* gene deleted versions of the bacmid backbone for expression of all four target proteins (Fig. 5AB). This observation is in agreement with previous studies (Berger et al., 2004; Hitchman et al., 2010a) supporting the beneficial effect of these baculoviral gene deletions for heterologous protein expression. This effect may be due to accumulation of chitinase (*chiA)* in the endoplasmic reticulum (Thomas et al., 1998) which may interfere with recombinant proteins targeted to the secretory pathway of the cells (Possee et al., 1999). Its deletion favours the production of secreted proteins (Possee et al., 2008). Since it also acts as activator of the baculoviral protease *v-cath*, which itself has a negative effect on recombinant protein stability, double deletions of *v-cath/chiA* have been introduced into many commonly used baculoviruses (Berger et al., 2004; Kaba et al., 2004). Additional gene deletions p10, p26, p74 have been shown to further enhance protein expression (Hitchman et al., 2010b). The labs (4 out of 14) using non-modified baculovirus performed with only limited success in expression of the selected set of proteins (Fig. 5).

Furthermore, a clear difference in performance was revealed by directly comparing the difference in expression levels (Fig. 3A-D) and protein yields (Fig. 4AB) between laboratories using transposition-based gene integration (Bac-to-Bac®, MultiBac® and EMBacY) and laboratories using recombination-based transfection (*flash*BAC™, *flash*BACULTRA™, ProGreen™ and DefBac). Most high and medium performing labs can

be found in the transposition-based integration group (M, H, X, E) whereas most low performing labs (C, D, K, V) are among the users of recombination-based transfection. Plotting the scored overall performance of all groups using Bac-to-® MultiBac® and EMBacY versus the users of *flash*BAC™, *flash*BACULTRA™, ProGreen™ and DefBac shows a two-fold higher performance of the transposition-based gene integration group (Fig. 6AB). This comparison was intentionally planned to be unbiased, meaning that all other parameters used in this BEVS study such as cell line, medium, expression conditions (Table 2) have not been standardized. Therefore, the observation that users of transposition-based integration perform better is a significant enough trend to justify several follow-up experiments.

3.5. Follow-up study to identify key parameters for high performance

To further strengthen the impact of the type of BEVS (transposition based gene integration versus recombinant-based transfection) on protein expression in insect cells, a follow-up study using standardized parameters was initiated. In the initial 13 lab-study, most medium and high performers (lab E, H, M and X) used the EMBacY baculovirus, which carries the *v-cath*/*chiA* deletion plus an additional integrated copy of YFP as a fluorescent marker. The purpose of the follow-up experiments was to compare protein expression by low and medium performers between EMBacY and their respective recombinant-based transfection BEVS keeping all other parameters such as cell line, medium, incubation temperature, etc constant. Labs D (*flash*BAC™), F (*flash*BACULTRA™), and V (ProGreen™) volunteered to participate. In addition, one low performing lab among the transposition-based integration group joined this study: lab L had used the non-modified Bac-to-Bac® BEVS, which was now compared with the Δ *v-cath*/*chiA* EMBacY baculovirus. Three genes of the prior study were selected for the follow-up study. All participants were provided with recombinant EMBacY bacmid DNA of constructs Dicer-2, ABL1 and FMRP and asked to proceed

according to the initial study, except using production volumes of only 20 mL. Protein expression levels in total lysates were analyzed as described before. Representative results from lab D and F are illustrated in Fig. 6A and B. Triplicate expression samples from lab D using *flash*BAC™ or EMBacY baculoviruses freshly prepared for that study under identical expression conditions were analysed by Easy SDS-PAGE and Cy5 quantification, as shown in Fig. 6A. The use of EMBacY instead of *flash*BAC™ increased expression levels of Dicer-2 from undetectable to 17% of total protein, for ABL1 from undetectable to 19% of total protein and for FMRP from 4% to 29% of total protein. A time-course of expression of Dicer-2, ABL1 and FMRP performed by lab F using either *flash*BACULTRA™ or EMBacY baculovirus is shown in Fig. 6B. Total lysates of cells were analysed and quantified by SDS-PAGE and the Bio-Rad Chemi-Doc XRS+ imaging system. In agreement with the initial study, lab F having high expression levels of 38% FMRP with *flash*BACULTRA™, could achieve only a slight increase to 41% using the EMBacY. However, EMBacY had a major impact on the difficult-to-express Dicer-2 and ABL1 protein expression levels which could be increased from 17% to 47% and from 5% to 15%, respectively. This effect is irrespective of prior optimization of the individual expression conditions for each BEVS system. Rescue of performance with EMBacY was accordingly successful for labs V and L, as illustrated in Fig. 6C. Expression levels in total lysates of cells derived from both labs increased using the EMBacY baculovirus from undetectable to 46% for Dicer-2 (lab L), from undetectable to 32% for ABL1 (lab L) and from undetectable to 44% for FMRP (lab V). For lab L, this rescue most likely relies on the use of a Δ*v-cath*/*chiA* viral backbone.

Apart from the increase in expression depending on the type of BEVS, differences in expression levels of Dicer-2, ABL1 and FMRP between lab D, F, L and V in the range of 10% to 50% using the EMBacY baculovirus reveal that other parameters also affect performance, although to a lesser extent than the genetic phenotype of the baculoviral

expression vector. Further fine tuning the influence of cell line, medium, amount of virus, infection time, etc, on expression was beyond the scope of this study.

In summary, the follow-up study corroborates the finding from the initial 13 lab-study, that the use of a modified transposition-based baculovirus is favourable for expressing our selected set of target proteins. The proteins chosen for the study presented here are all intracellularly located; expressed at levels below 1 mg/L culture; include difficult-to-express proteins of high molecular weight like Dicer-2 (204 kDa) and ABL1 (126 kDa), have limited stability (Dicer-2 and ABL1) or solubility (FMRP). However, the results of lab R (*flash*BACULTRA^{TM}) and U (DefBac, Δ*v-cath /chiA*) show that the recombination-based transfection baculovirus also has the potential to achieve prosperous results in expressing Dicer-2, ABL1, FMRP and NS1-H. The most important remaining question is: why do most labs within this study using recombinant-based transfection only express limited amounts of this particular set of target proteins, even though 6 out of 7 are using baculovirus variants carrying deletions of Δ*chiA*, Δ*v-cath*/*chiA* or Δ*v-cath /chiA/p10/p74/p26*, shown to be beneficial for protein expression as described above? The lack of expression of Dicer-2, ABL1, FMRP and NS1-H1, more or less pronounced in lab C, D, F, K, and V is obviously not due to basic cell culture problems, since the performance in selected labs could be restored by using EMBacY. Altogether, this suggests that the procedures that were followed to enable recombinant-based transfection were apparently sub-optimal rather than inherent differences in the baculoviruses. EMBacY uses Tn7 transposition of the target gene from the corresponding transfer vector into the baculovirus genome within *E. coli* cells already carrying the virus genome. Recombinant baculovirus is visualized by blue-white screening and the bacmid DNA can be further analyzed for correct gene integration by colony-PCR or DNA sequencing. This additional colony-picking step followed by downstream analysis to ensure transposition of the entire expression cassette makes this procedure more time-consuming, which is considered to be the major disadvantage compared to recombinant-based

transfection, but it excludes the risk of propagating virus in which insertion of the target gene is somehow compromised. Furthermore, YFP integrated into EMBacY allows for monitoring of virus production with high sensitivity using either fluorescence microscopy or spectrophotometry. Since YFP is under control of the very late polyhedrin polH promotor, it serves as an internal marker for the infection process and for onset of protein expression, as the gene for the heterologous protein is usually under control of the same polH promotor. The recombination-based transfection baculoviral expression system instead generates recombinant baculovirus directly in insect cells by co-transfecting transfer vector and bacmid DNA. This viral DNA is per se replication-incompetent, only allowing propagation of recombinant virus. In contrast to the time-consuming colony picking step in the transposon-based integration, this process is faster. However, since gene integration cannot be directly monitored, the quality of the recombined baculovirus for expressing the target gene may be less verifiable.

As a final conclusion, the study presented here may help new users of baculovirus-mediated protein expression in insect cells with the many choices to be made. The BEVS strategy, transposition-based integration or recombination-based transfection may have a major impact on the result. For the proteins expressed in this study, transposition-based integration was favourable and resulted in substantially improving the expression levels in the benchmarking labs. Basically, it is highly recommended to identify the most appropriate BEVS for a given target protein or protein family and then further optimize and fine-tune expression with cell lines, media, expression conditions etc. Moreover, the protocol of choice will have to fulfill several additional criteria as compatibility with high throughput techniques, scalability and downstream processes. Eventually, there might be no one-fits-all approach and the individual setup will be a compromise between optimal output and practical restrictions.

Figure Legends

Fig. 1. Design of the study

Fig. 2. Analysis of protein expression levels of all four proteins shown for three representative participants L, M and R. Blue arrowheads indicate full-length target proteins, red arrowheads highlight a Dicer-2 degradation product. (A) Total lysates of non-infected control cells and cells infected with the respective baculoviruses were loaded on 13.5% Amersham Easy SDS-PAGE gels, visualized by Cy5 staining and quantified by the Amersham WB software. Expression levels are indicated as [%] of total cellular protein. Samples labelled with "nd" were below detection level. (B) Total lysates of non-infected control cells and cells infected with the respective baculoviruses were separated via capillary electrophoresis and detected with primary anti-penta-His antibody (Qiagen) and secondary HRP-labeled anti-mouse antibody using the Peggy™ Simple Western system (Protein Simple).

Fig. 3. Comparison of protein expression levels of Dicer-2 (A), ABL1 (B), FMRP (C) and NS1-H1 (D) from all participants grouped in two parts: on the left users of the transposition-based integration systems (lab E, F, H, I, L, M, X), on the right users of recombination-based transfection systems (lab C, D, F, K, R, U, V). MB MultiBac®, fB *flash*BAC™ , Db DefBac, PG ProGreen™. Datasets from Easy SDS-PAGE and Simple Western are displayed for all constructs except for Dicer-2 and ABL1 from lab X which were analyzed only by Easy SDS-PAGE (see * in graphs). In order to compare the different datasets side-by-side, the highest value within each dataset was set to 100% performance and all values within the respective dataset related to it.

Fig. 4 Results of protein enrichment by one-step IMAC chromatography analyzed on 13.5 % Amersham Easy SDS-PAGE gels. MB MultiBac®, fB *flash*BAC™ (A) Imidazole eluates of Dicer-2, ABL1, FMRP and NS1-NH1 proteins shown for three representative participants M, R and U. Protein purity is indicated as [%] based on Cy5 staining and quantification by

Amersham WB software. Respective bands are highlighted with blue arrowheads. (B) Comparison of protein yields of Dicer-2, ABL1, FMRP and NS1-NH1 from all participants. Protein yields have been derived from Bradford protein quantification corrected by protein purity based on Cy5 staining as shown in Fig. 4A. In order to compare protein yields and expression levels side-by-side, the highest protein yield value was set to 100% and all other values were normalized to it. Results are grouped in two parts: on the left users of the transposition-based integration systems (lab E, F, H, I, L, M, X), on the right users of recombination-based transfection systems (lab C, D, F, K, R, U, V). Labs within each group were ordered according to performance.

Fig. 5: Scored performance of users of the non-modified baculoviruses (Lab $F_{Bac\text{-}to\text{-}Bac}$, I, L and V) versus users of the Δ*chiA*, Δ*v-cath*/*chiA* or Δ*v-cath/chiA/p10/p74/p26* deleted baculoviruses (Lab C, D, $F_{flashBAC}$, K, M, H, X, E, R, U) for both groups: transposition-based integration (lab E, F, H, I, L, M, X) and recombination-based transfection (lab C, D, F, K, R, U, V). *Scores represent the sum of performances [%] divided by number of labs included in the respective group with regard to protein expression levels (derived from data displayed in Fig. 3) and protein yields (derived from data displayed in Fig. 4). Both groups are represented with 7 labs, thus equally weighted.

Fig. 6: Comparison of protein expression levels of Dicer-2, ABL1 and FMRP using either the BEVS system from the 1st study or EmBacY. (A) Lab D sample triplicates of total lysates expressed with *flash*BAC™ (virus freshly prepared for the 2nd study) or with EmBacY baculovirus analyzed by Easy SDS-PAGE. Expression levels are indicated as [%] of total cellular protein. Expression was performed in Hi5 cells at 27 °C for 72 h at virus dilutions of 1:350 – 1:150 optimized for each individual construct in 125 mL flasks. Blue arrowheads highlight the overexpressed proteins. (B) Lab F time-course of expression using either *flash*BACULTRA™ or EMBacY in Hi5 cells in ESF 921 medium at 21 °C, collected after

72, 96, 120, 144 hours post-infection (P.I. hrs) and analyzed by SDS-PAGE. Quantification was performed with the Bio-Rad Chemi-Doc XRS+ imaging system. (C) Expression levels as [%] of total cellular protein derived from quantitative Easy SDS-PAGE for labs D, L and V or Bio-Rad Chemi-Doc XRS+ imaging system for lab F. Expression conditions used were identical to the 1st study (Table 2) except use of virus instead of BIICs in lab L.

References

Berger, I., Fitzgerald, D.J., Richmond, T.J., 2004. Baculovirus expression system for heterologous multiprotein complexes. Nat Biotechnol 22, 1583-1587.

Berger, I., Garzoni, F., Chaillet, M., Haffke, M., Gupta, K., Aubert, A., 2013. The multiBac protein complex production platform at the EMBL. J Vis Exp, e50159.

Bieniossek, C., Richmond, T.J., Berger, I., 2008. MultiBac: multigene baculovirus-based eukaryotic protein complex production. Curr Protoc Protein Sci Chapter 5, Unit 5 20.

Bjerneld, E.J., Johansson, J.D., Laurin, Y., Hagner-McWhirter, A., Ronn, O., Karlsson, R., 2015. Pre-labeling of diverse protein samples with a fixed amount of Cy5 for sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Anal Biochem 484, 51-57.

Colicelli, J., 2010. ABL Tyrosine Kinases: Evolution of Function, Regulation, and Specificity. Science signaling 3, re6-re6.

Cox, M.M., Hollister, J.R., 2009. FluBlok, a next generation influenza vaccine manufactured in insect cells. Biologicals 37, 182-189.

Deschuyteneer, M., Elouahabi, A., Plainchamp, D., Plisnier, M., Soete, D., Corazza, Y., Lockman, L., Giannini, S., Deschamps, M., 2010. Molecular and structural characterization of the L1 virus-like particles that are used as vaccine antigens in Cervarix, the AS04-adjuvanted HPV-16 and -18 cervical cancer vaccine. Hum Vaccin 6, 407-419.

Fan, H., Ju, C., Tong, T., Huang, H., Lv, J., Chen, H., 2007. Immunogenicity of empty capsids of porcine circovius type 2 produced in insect cells. Vet Res Commun 31, 487-496.

Hitchman, R.B., Possee, R.D., Siaterli, E., Richards, K.S., Clayton, A.J., Bird, L.E., Owens, R.J., Carpentier, D.C., King, F.L., Danquah, J.O., Spink, K.G., King, L.A., 2010a. Improved expression of secreted and membrane-targeted proteins in insect cells. Biotechnol Appl Biochem 56, 85-93.

Hitchman, R.B., Possee, R.D., Crombie, A.T., Chambers, A., Ho, K., Siaterli, E., Lissina, O., Sternard, H., Novy, R., Loomis, K., Bird, L.E., Owens, R.J., King, L.A., 2010b. Genetic modification of a baculovirus vector for increased expression in insect cells. Cell Biol Toxicol 26, 57-68.

Kaba, S.A., Salcedo, A.M., Wafula, P.O., Vlak, J.M., van Oers, M.M., 2004. Development of a chitinase and v-cathepsin negative bacmid for improved integrity of secreted recombinant proteins. J Virol Methods 122, 113-118.

Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., Carthew, R.W., 2004. Distinct Roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA Silencing Pathways. Cell 117, 69-81.

Possee, R.D., Thomas, C.J., King, L.A., 1999. The use of baculovirus vectors for the production of membrane proteins in insect cells. Biochem Soc Trans 27, 928-932.

Possee, R.D., Hitchman, R.B., Richards, K.S., Mann, S.G., Siaterli, E., Nixon, C.P., Irving, H., Assenberg, R., Alderton, D., Owens, R.J., King, L.A., 2008. Generation of baculovirus vectors for the high-throughput production of proteins in insect cells. Biotechnol Bioeng 101, 1115-1122.

Rustandi, R.R., Loughney, J.W., Hamm, M., Hamm, C., Lancaster, C., Mach, A., Ha, S., 2012. Qualitative and quantitative evaluation of Simon, a new CE-based automated Western blot system as applied to vaccine development. Electrophoresis 33, 2790-2797.

Thomas, C.J., Brown, H.L., Hawes, C.R., Lee, B.Y., Min, M.K., King, L.A., Possee, R.D., 1998. Localization of a baculovirus-induced chitinase in the insect cell endoplasmic reticulum. J Virol 72, 10207-10212.

Till, S.M. 2010. The developmental roles of FMRP. Portland Press Limited.

van Oers, M.M., Pijlman, G.P., Vlak, J.M., 2015. Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. Journal of General Virology 96, 6-23.

Acknowledgements

Helmholtz Protein Sample Production Facility (PSPF) thanks Anke Samuels for excellent technical support during the experiments. Lund Protein Production Platform (LP3) would like to thank its staff members Ewa Krupinska and Anna Rasmussen for excellent technical assistance in executing this study at our facility. The Max-Planck Institute of Biochemistry, Structural Cell Biology Department thanks Elfriede Eppinger for excellent technical assistance in performing experiments.

Funding

Helmholtz Protein Sample Production Facility (PSPF): This work was supported by the Helmholtz Protein Sample Production Facility (PSPF) and Instruct, part of the European Strategy Forum on Research Infrastructures.

Vienna Biocenter Core Facilities acknowledges funding from the Federal Ministry of Science, Research, and Economy (bm.wfw) and the City of Vienna.

Declaration of interest

Conflict of interest: none

4 intracellular target proteins
identical for both BEV systems

7 laboratories
E, F, I, L, M, H, X

in **pFastBac** for transposon-based integration

- Bac-to-Bac™
- MultiBac
- EMBacY

7 laboratories
C, D, F, K, R, U, V

in **pBac1** for integration by recombination-based transfection

- *flash*Bac™
- *flash*BacUltra™
- ProGreen™
- DefBac

Use of in-house routine protocols

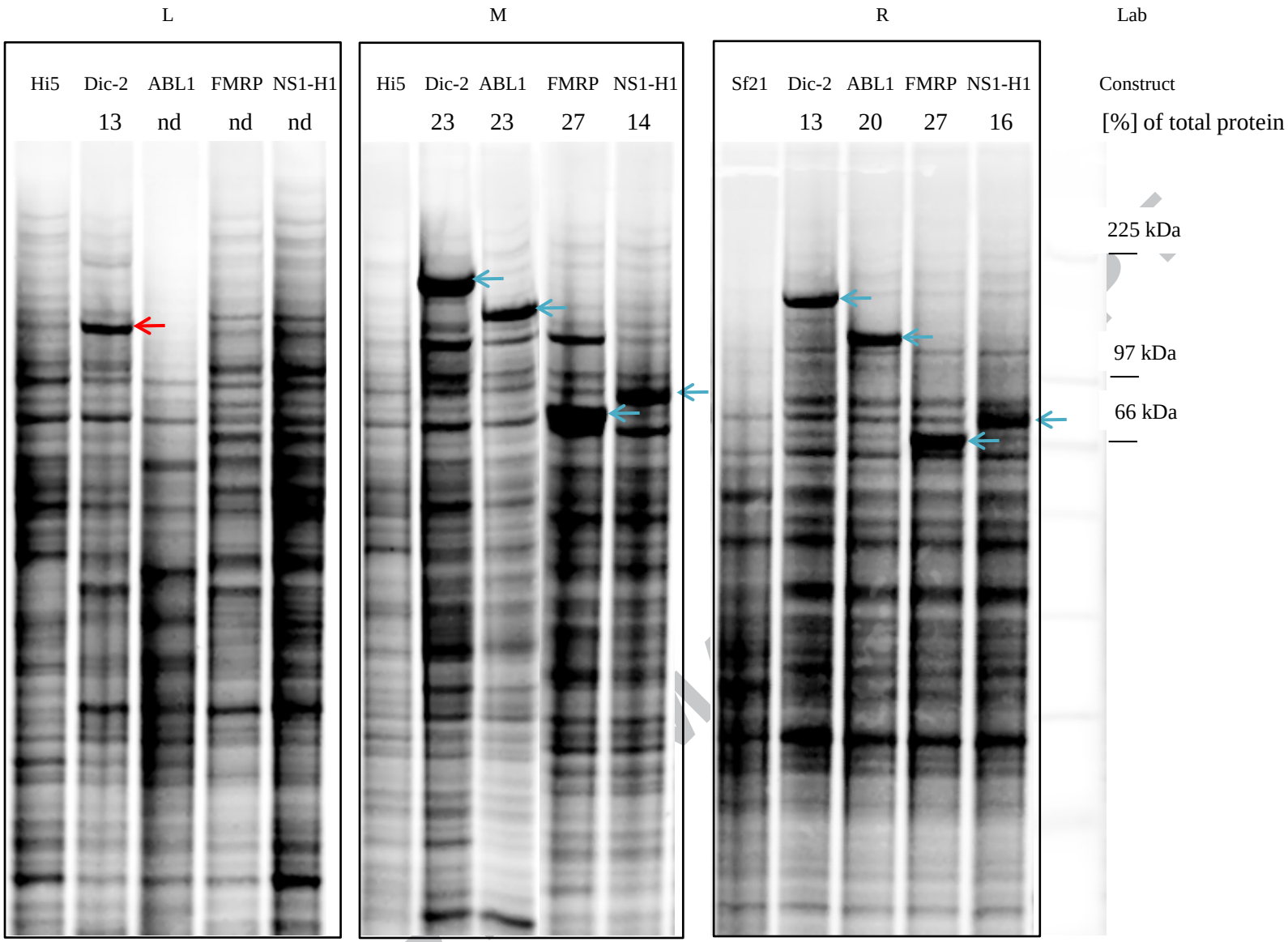
- Sub-cloning (if appropriate)
- Transfection and virus amplification
- Small scale expression
- 500 ml scale production of each construct

- ✓ cell densities and viabilities
- ✓ 3 x 500 µl cell pellets
- ✓ 2 x 10 ml cell pellets
- ✓ Control cells

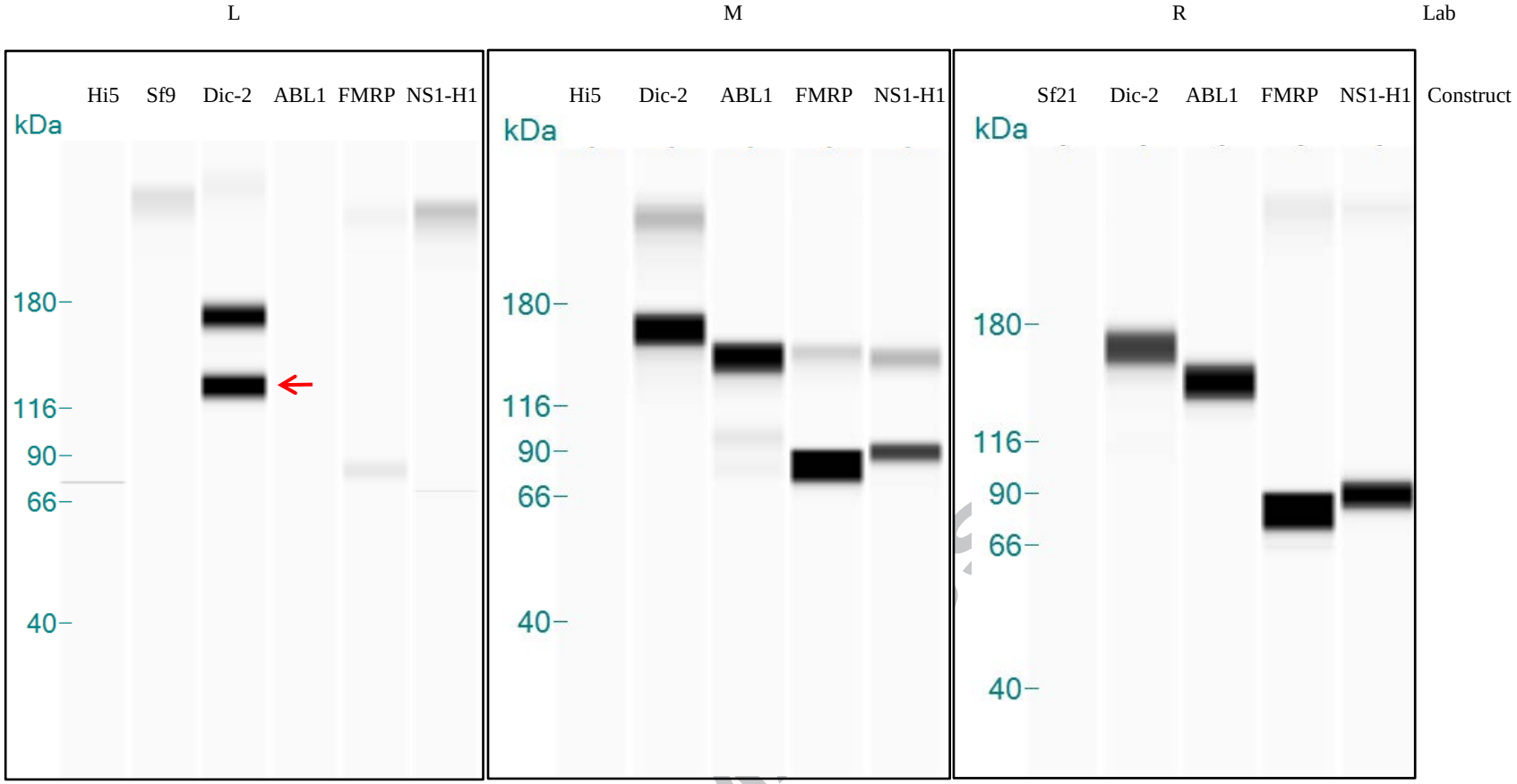
Centralized sample analyis

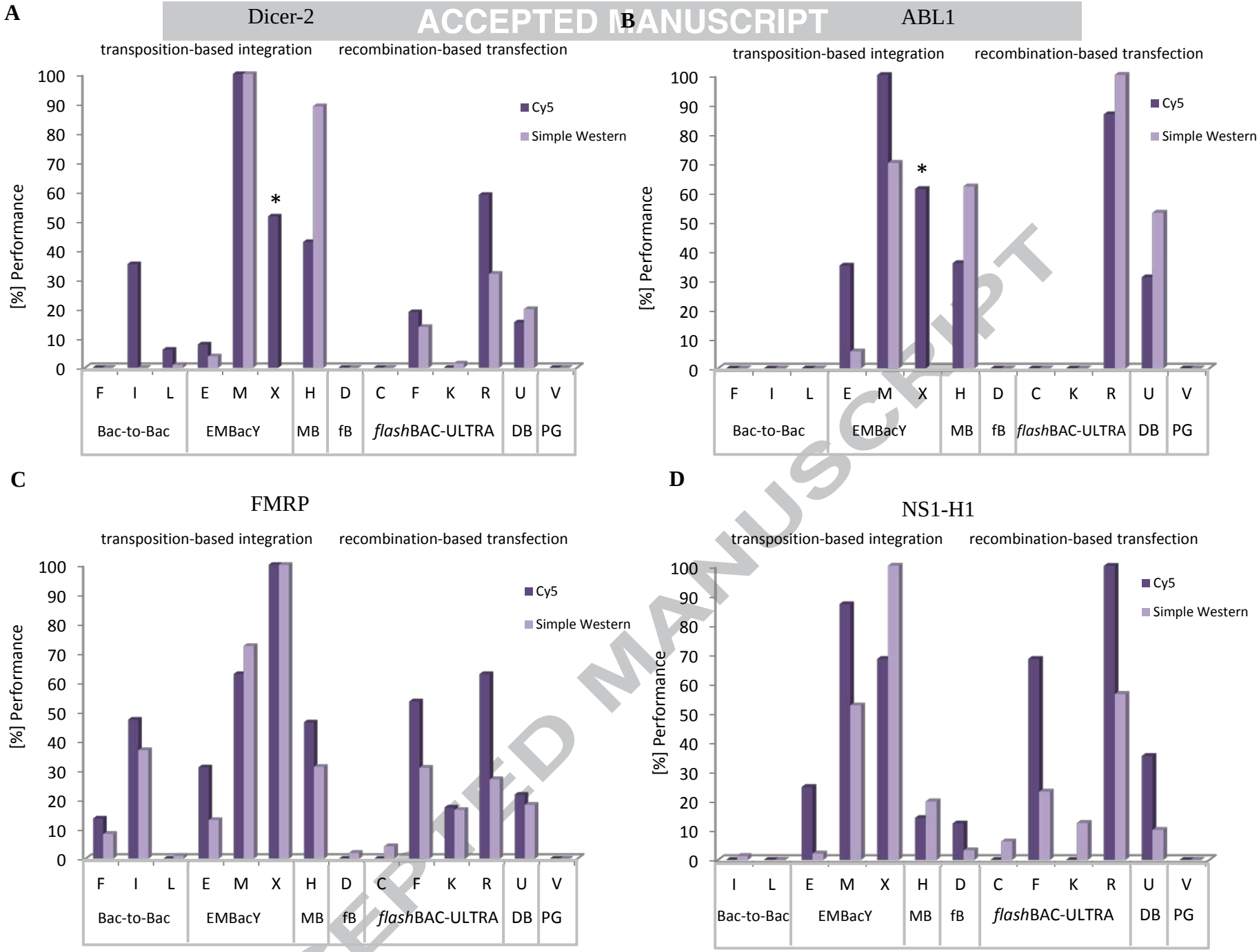
- Simple Western (VBCF)
- SDS PAGE (MPIB)
- Protein purification (MPIB)

A

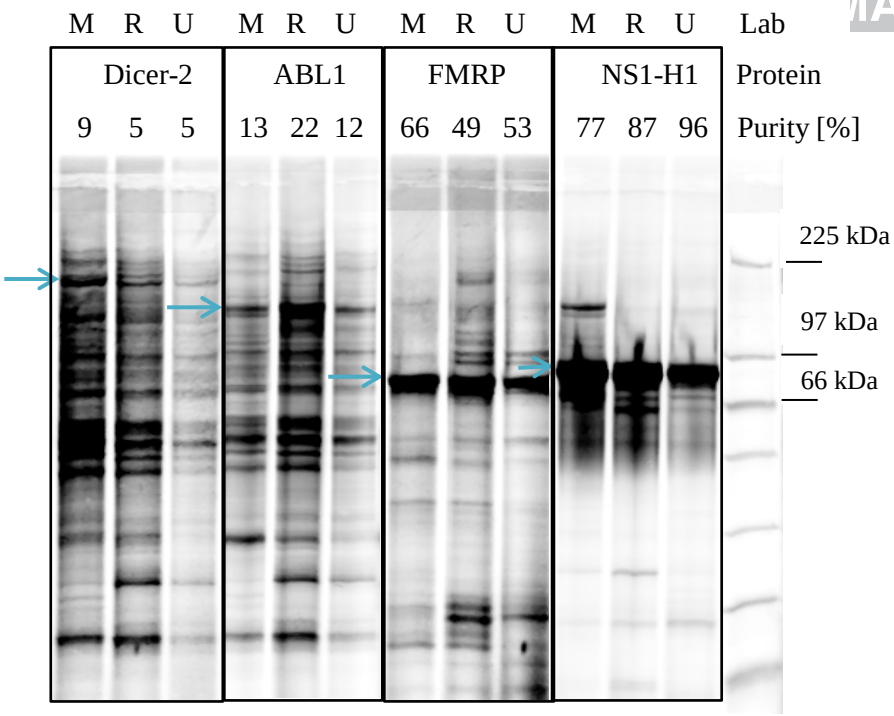


B

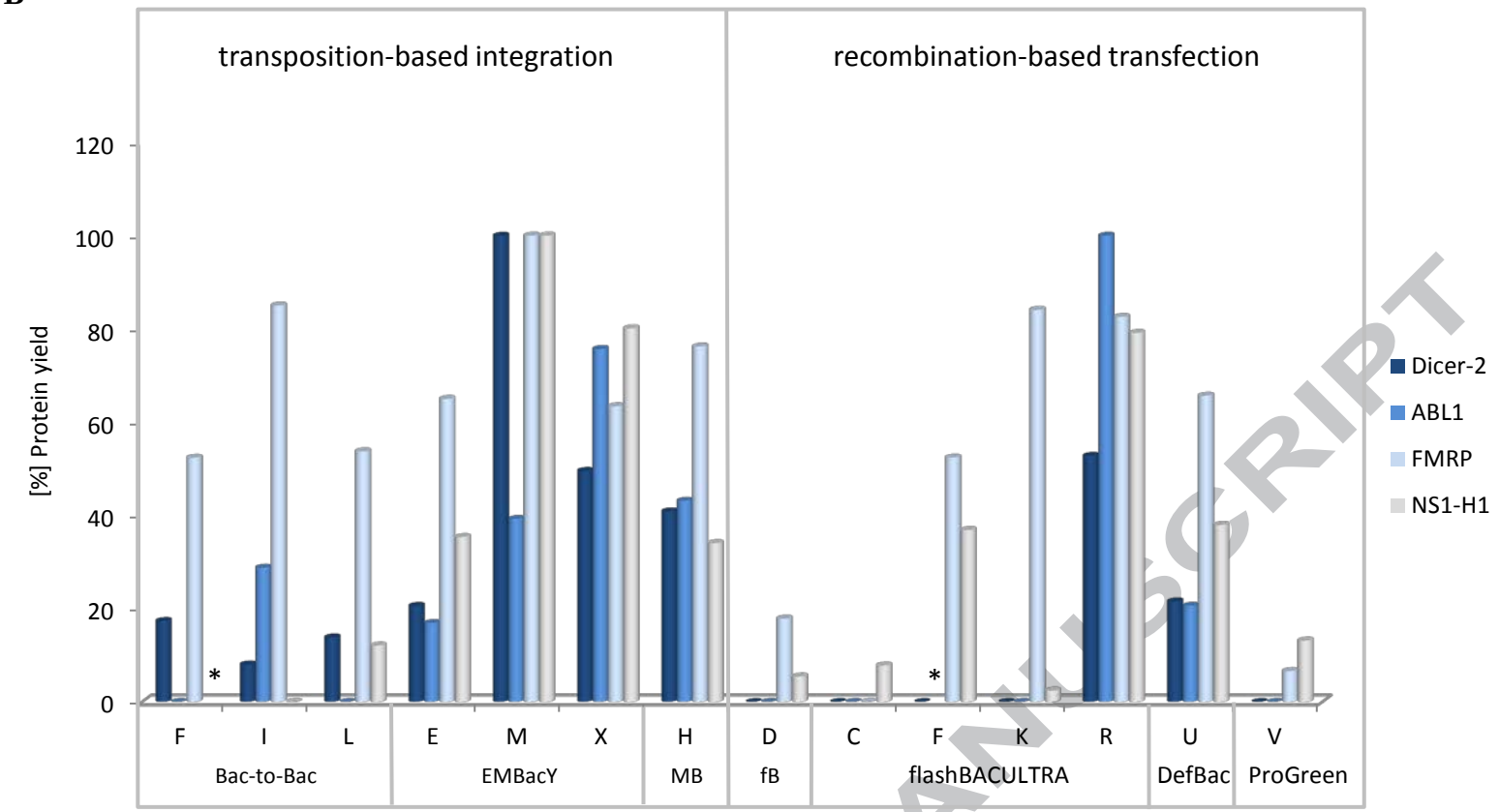




A

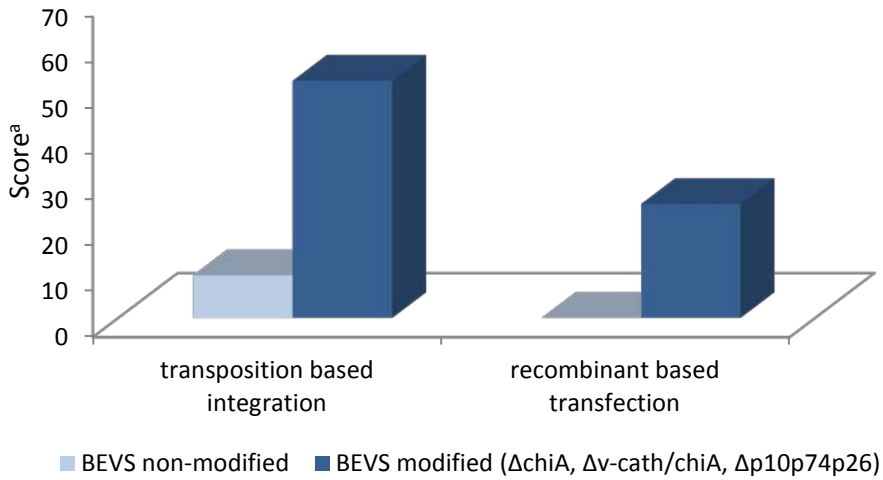


B



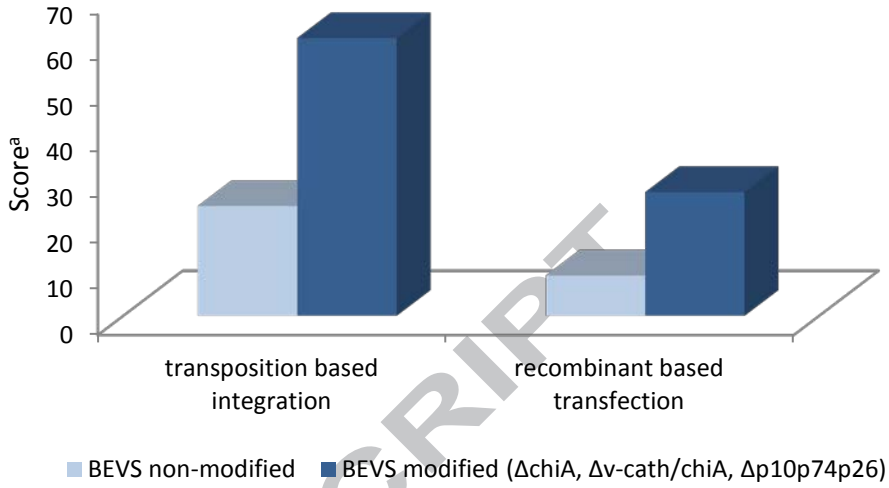
A

Expression level

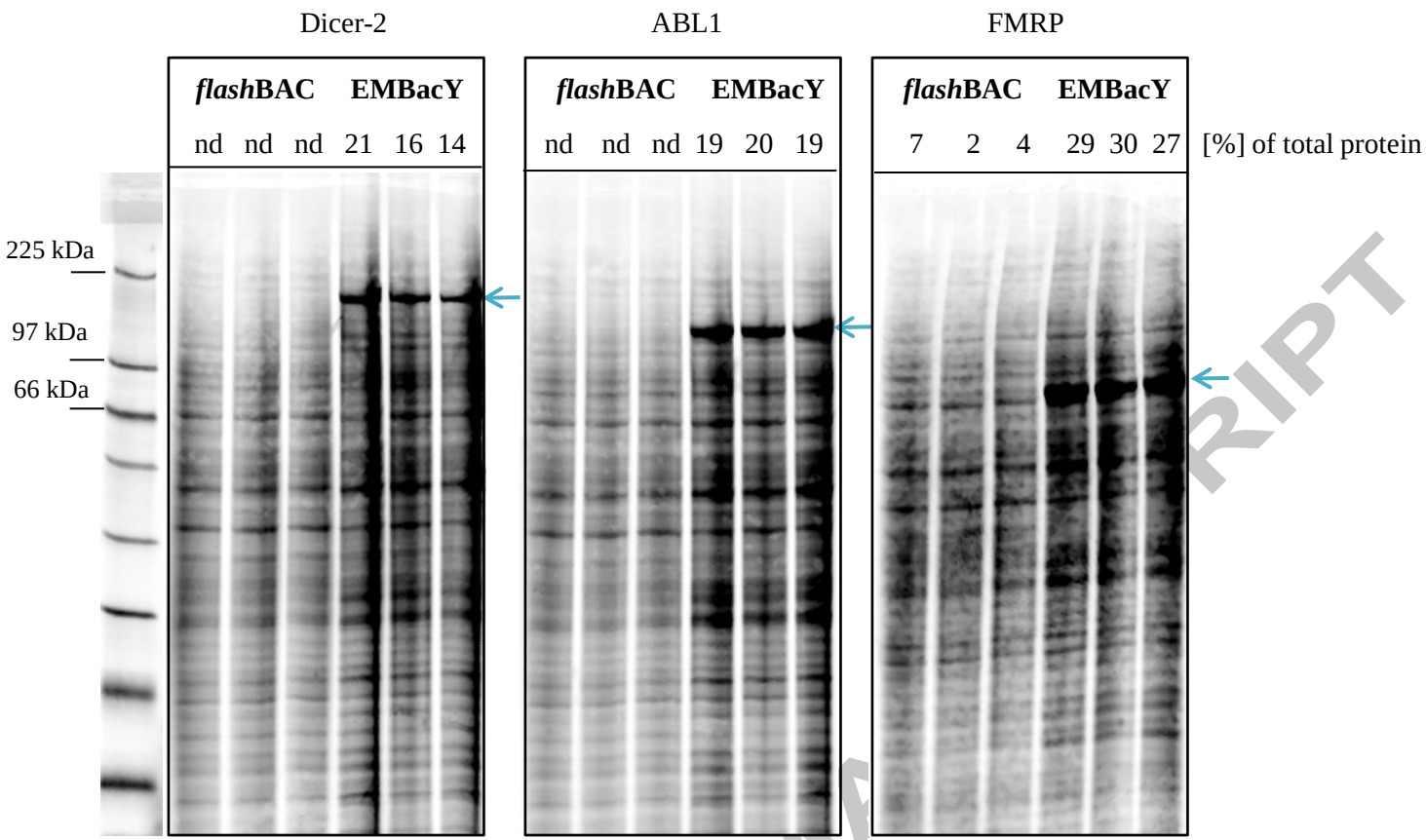


B

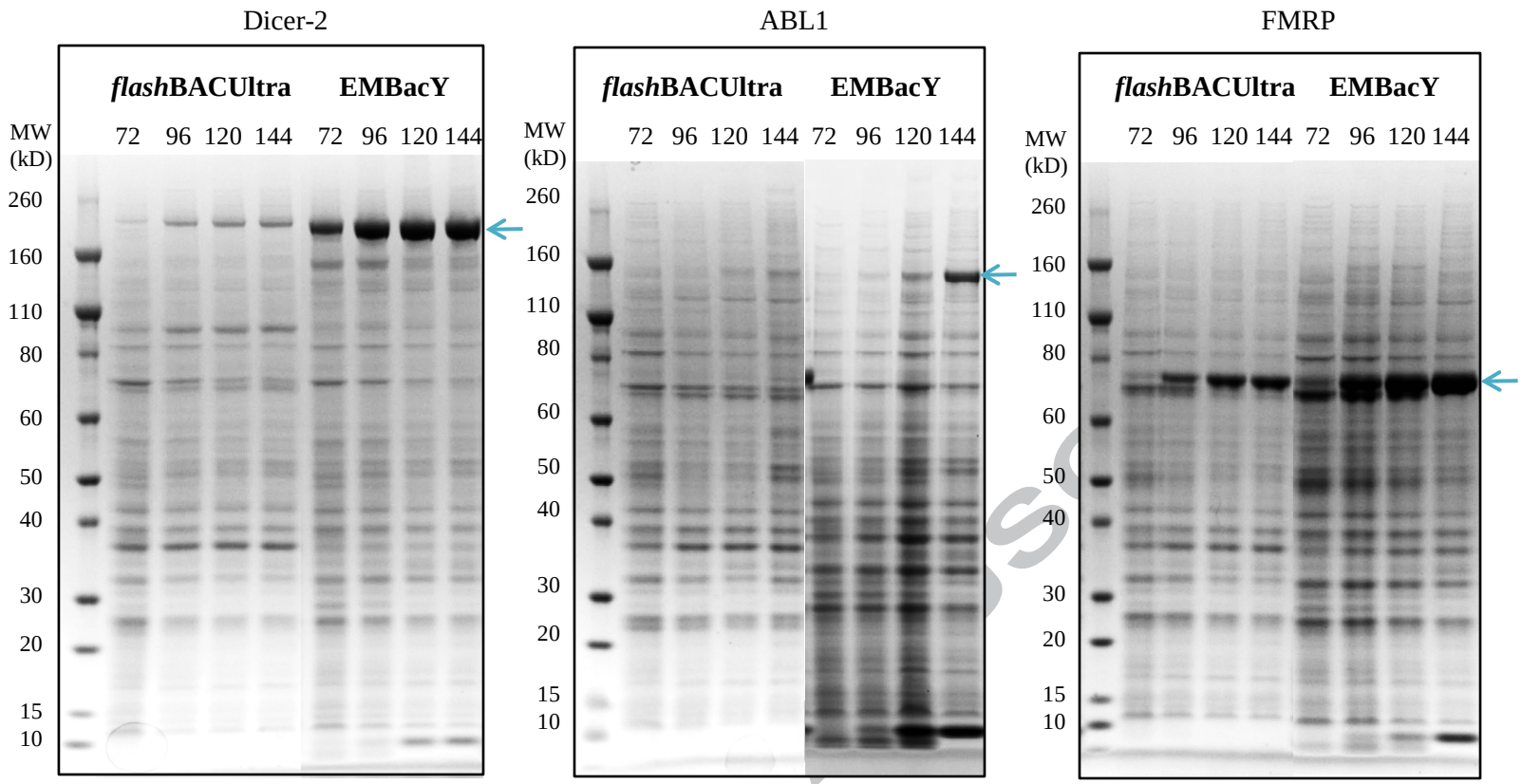
Protein Yield



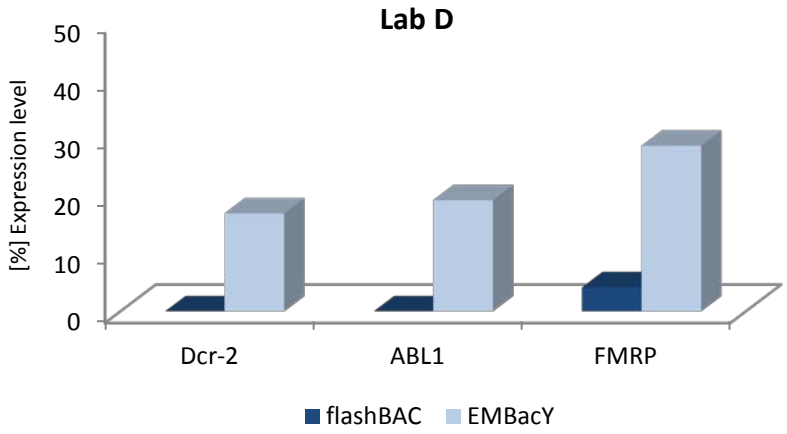
A

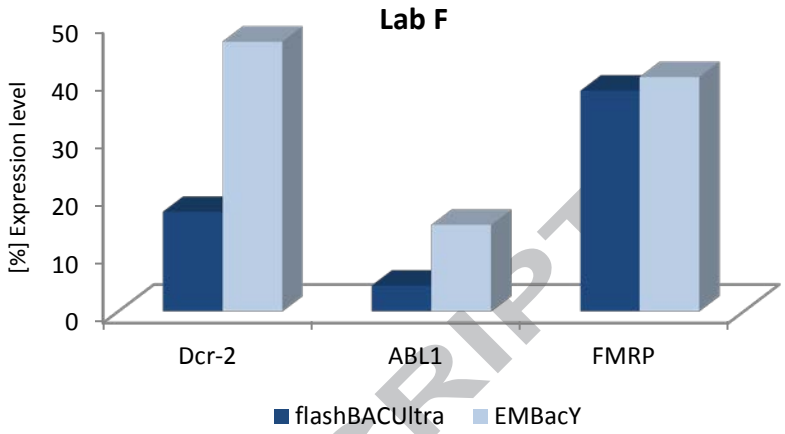


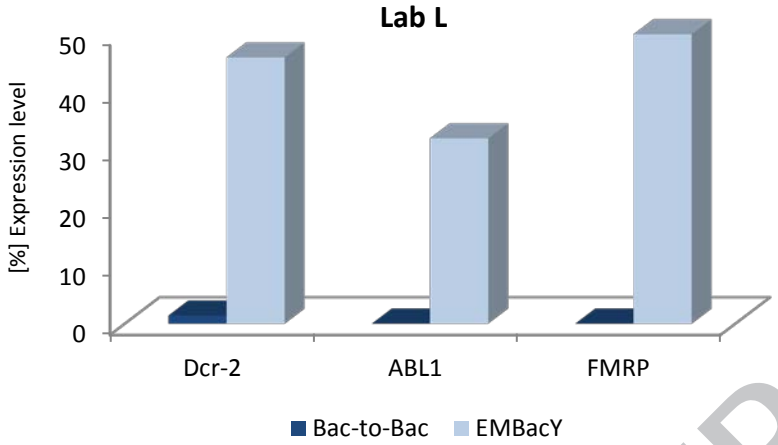
B



C







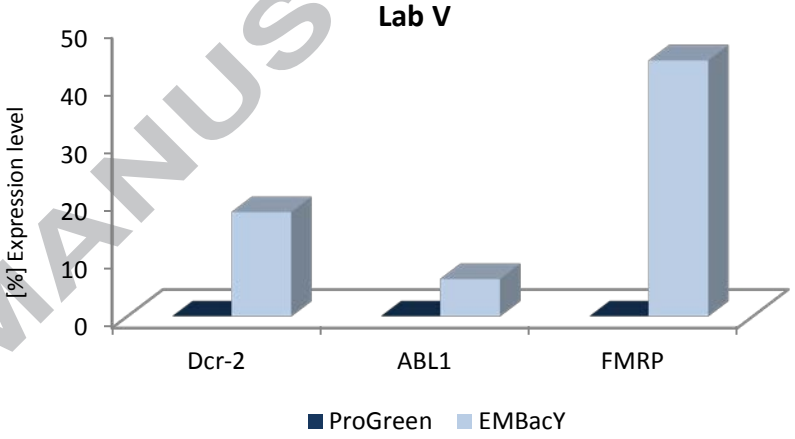


Table 1

Protein candidates in benchmarking exercise

Number	Protein	UniProt entry	MW [kDa]	Tag	Typical yield* [mg/L]
1	mDicer-2	A1ZAW0	203.9	N-His_6-TEV-Flag	< 1
2	hcABL1wt	P00519	126.4	N-His_6-TEV	< 1
3	hFMRP	P35922-2	69.9	N-His_6-TEV	< 1
4	vNS1-H1	P03133	78.4	N-His_6 - TEV	< 1

*Typical yield after protein purification.

Table 2 Expression parameters and conditions

Lab	BEVS	Gene deletions of BEVS	Cell line	Medium	Amount virus used	Cell density at infecti	Time of harvest	Incubation temp [°C]	Production Flask
C	*flash*BACUltra™	Δ*v-cath/chiA/p10/*	Sf21	EX-CELL® 420	1:10	0.6	48	28	3 L Corning
D	*flash*BAC™	Δ*chiA*	Hi5/S9	SFX-Insect	1:100 - 1:35	1	72-96	27	3 L Corning
E	EMBacY	Δ*v-cath/chiA*	Sf9	Sf-900™ II	1:2000	0.9	72	27	1.8 L Fernbach
F	Bac-to-Bac®	none	Hi5	Sf-900™ II	1:66	1.5	96-120	21	2.8 L Fernbach
F	*flash*BACUltra™	Δ*v-cath/chiA/p10/*	Hi5	Sf-900™ II	1:66	1.5	72-144	21	2.8 L Fernbach
H	MultiBac®	Δ*v-cath/chiA*	Sf21	Sf-900™ III	1 :100-1 :18	0.35 – 1.4	72-120	27	3 L glass EM
I	Bac-to-Bac®	none	Sf21	Insect-XPRESS™	1:50	1	72	28	2 L glass EM
K	*flash*BACUltra™	Δ*v-cath/chiA/p10/*	Hi5/Sf9	EX-CELL® 405/ 420	1:130-1:33	1.3 -3	48-96	27	2 L glass EM
L	Bac-to-Bac®	none	Hi5, Sf9	EX-CELL® 420	1:1000 - 1:250	1	72	26	1.8 L Thomson,
M	EMBacY	Δ*v-cath/chiA*	Hi5	Sf-900™ II	1:125	1	68-72	26	3 L Corning
R	*flash*BACUltra™	Δ*v-cath/chiA/p10/*	Sf21	proprietary	1:33	1,5 - 2	48	27	2 L glass EM
U	DefBac*	Δ*v-cath/chiA*	Sf9	ESF 921™	1:100	1	72-96	27	1.8 L Fernbach
V	ProGreen™	none	Hi5	ESF 921™	1:30	1	48-72	27	2 L Fischerbra
X	EMBacY	Δ*v-cath/chiA*	Hi5	ESF 921™	1:500	1	72	27	2 L glass EM +

* uses recombination based transfection; manuscript in preparation by Lab U.