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CELL-FREE EXPRESSION/ REPORTED SYSTEM, DEVELOPED

D4.3

JENNIFER CHOW AND WOLFGANG STREIT

UHAM

Department of Microbiology and Biotechnology, Ohnhorststrasse 18, 22609, Hamburg, Germany

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Authors:	UHAM (Wolfgang R. Streit, Jennifer Chow, Pablo Pérez García), CSIC (Manuel Ferrer, Patricia Molina)
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Contact details:	Wolfgang Streit (wolfgang.streit@uni-hamburg.de), Jennifer Chow (jennifer.chow@uni-hamburg.de), Manuel Ferrer (mferrer@icp.csic.es)

Summary

Abbreviations

Aa, Amino acid
ATP, Adenosine triphosphate
DEPC, Diethylpyrocarbonate
DTT, Dithiothreitol
FACS, Fluorescence-activated cell sorting
HCl, Hydrogen chloride
HTS, High Throughput Screening
ivTT, *in vitro* transcription and translation
LB, Luria Bertani
MHET, Mono-(2-hydroxyethyl) terephthalate
NAD, Nicotinamide adenine dinucleotide
PCL, Polycaprolactone
PCR, Polymerase chain reaction
PEP, Phosphoenolpyruvate
PET, Polyethylene terephthalate
p-NP, *p*-Nitrophenol
PPase, Thermostable inorganic pyrophosphatase
RNA, Ribonucleic acid
RNAP, RNA-polymerase
rNTP, Ribonucleoside tri-phosphate (rATP, rCTP, rGTP, rUTP)
TB, Transcription buffer
TPA, Terephthalic acid
tRNA, Transfer RNA

1. Scope of Deliverable

This deliverable consists in a cell-free expression system, to be made available to partners, in which enzymes are produced and enzymatic activities detected in a high-throughput manner by skipping the step of recombinant expression. This deliverable is accompanied by a report detailing the design and functioning of the cell-free expression system. The protocol of use is available in the internal FuturEnzyme repository through this link (credentials: Dr. Patricia Molina Espeja, %wf%G85uzu6Wq3Isa$84L35h).

2. Introduction

Metagenomic screening is a widely applied approach for screening novel enzymes with a potential for biotechnological applications. The traditional method of metagenomic screening is based on the functional analyses of heterologously expressed environmental genes in a suitable host, which is the bottleneck of this method. To avoid limitation from the clone-dependent system, an *in vitro* expression technology has been developed. In the beginning, genes encoding the putative enzymes of choice have to be available. Mostly bioinformatic sequence-based searches deliver these genes. Either they can be amplified by PCR from a metagenome or, if the corresponding (meta-) genomic DNA is not available or the PCR is not successful, the gene sequence has to be synthesized commercially. The candidate genes are then expressed *in vitro* with an RNA polymerase for transcription and a translation machinery from special cell extract (**Fig. 1**). The function analyses can be done directly with *in vitro* expressed candidate proteins when a sensitive screening method is available. There are several advantages to perform enzyme screenings with such a cell-free technology. First, it is much faster than the traditional metagenomic screening methods. Because it is not necessary to construct libraries with huge numbers of clones to cover a large amount of genomic information, it saves manpower and materials. Normally, it takes only one day to express a protein with this *in vitro* expression method. Thus, it saves a lot of time compared to bacterial expression systems. Second, the screening can be performed not only within private metagenomic datasets but also within publicly available databases. Larger databases provide a higher chance to find a proper enzyme suitable for later application. Third, the major bottleneck of the traditional function-based metagenomics screening is the heterologous expression in suitable hosts to get the sufficient yield of functional enzymes. Common problems related to heterologous expression are e.g. low induction rate, inclusion body formation, different codon usage, etc. However, such problems can be avoided with the *in vitro* expression system. Finally, with the help of automatic pipetting robots, an *in vitro* metagenomic screening can be performed in high-throughput manner.

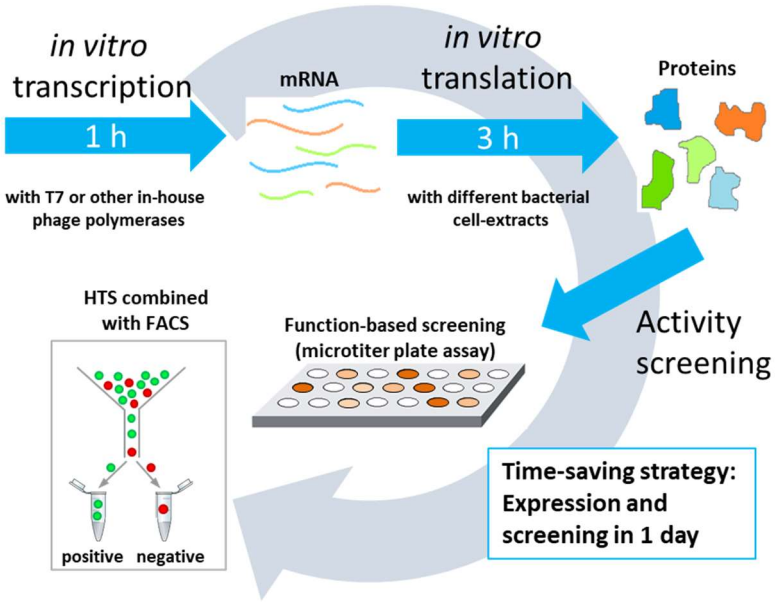

Figure 1. Overview on the general workflow of the cell-free *in vitro* transcription and translation system.

The cell-free expression system presented in this deliverable can be applied to almost all enzymes classes important in the FuturEnzyme project, such as amylases, lipases, proteases, cutinases, lactonases, and oxidoreductases.

For the *in vitro* transcription step, either a commercial T7-RNA-polymerase can be used or e.g. the EM1-RNAP found, produced and characterized at UHAM. The cell-free extract can be produced from RNase- and protease-deficient *Escherichia coli* strains or other suitable bacteria. For the purposes within the project, sensitive enzyme assays are usually carried out in a microtiter plate scale of up to 250 µL of volume. A possible way to expand this system to high-throughput in the future is to combine it with a FACS-based microfluidic droplet sorter.

In this report, we present a method for metagenomic screening via the *in vitro* expression system using an enzyme called polyethylene terephthalate (PET) esterase as example, because its relevance in the context of FuturEnzyme for textile recycling. PET esterases are related to cutinases and can cleave PET polymers into several small soluble compounds, such as bis-(2-hydroxyethyl) terephthalate (BHET), mono-(2-hydroxyethyl) terephthalate (MHET), terephthalic acid (TPA) and ethylene glycol (EG). PET esterases find a potential application, among others, in washing detergents to decrease the amount of micro-and nanoplastic particles in the waste water that are derived from washing polyester fabrics.

3. Protocols for *in vitro* transcription and translation

3.1. Preparation of DNA template

List of required materials:

- Primer pair: The forward primers should either include a promotor sequence recognizable for the RNAP or the forward primer should bind upstream before the promotor sequence of a vector containing the desired gene.
- Phusion High-Fidelity PCR Master Mix (2x) (Thermo Scientific, Waltham, MA, USA) or similar DNA-polymerase for PCR.
- NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Düren, Germany) or similar.
- NanoDrop 2000 Spectrophotometer (Thermo Scientific) or similar.

Usually, candidate genes are optimized for expression in *E. coli,* synthesized and cloned into a pET-21a(+) vector (Novagen, Madison, WI, USA). Then, the enzyme candidate genes in pET-21a(+) are amplified by PCR, because linear DNA fragments result in higher mRNA yields in the *in vitro* transcription.

The promoter recognized by EM1 RNAP can be introduced to the upstream of PET esterase candidate gene with the forward primer EFN4HR8C_Downstr(24-41)_pET (5'-ATAAGATCTTCAGAAGTCACACTATAA GGGAATTGTGAGCGGATAAC-3'; promotor sequence underlined). As reverse primer, pET-rev (5'-TCCGGATATAGTTCCTC-3') was used.

Pipette the PCR reaction as indicated in **Table 1**:

Table 1. PCR reaction mix for the generation of amplified DNA templates.

Components	Amount
Plasmid pET-21a(+)-enzyme candidate gene	0.25 µL
Phusion High-Fidelity PCR Master Mix (2x)	12.5 µL
Primer EFN4HR8C_Downstr(24-41) (10 µM)	2.5 µL
Primer pET-rev (10 µM)	2.5 µL
RNase-free water	Add to 25 µL

Set up PCR cycles as indicated in **Table 2**:

Table 2. PCR programm using EFN4HR8C_Downstr(24-41) and pET-rev primer.

Cycle step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	55°C	20 sec	
Extension	72°C	20 sec	
Final extension	72°C	3 min	1
Hold	4-10°C	∞	

Purify the amplified PCR products *e.g.* with a NucleoSpin® Gel and PCR Clean-up Kit according the manufacturer's instruction. Check the size of the DNA template with DNA gel electrophoresis and quantify the DNA amount using a NanoDrop 2000 Spectrophotometer. Take 1 µg of purified DNA for one *in vitro* transcription reaction.

3.2. *In vitro* Transcription

List of required materials:

- EM1 RNAP: self-made single subunit DNA-dependent RNA polymerase or other commercial RNA polymerases (*e.g.,* T7 RNA polymerase from Thermo Scientific).
- Thermostable inorganic pyrophosphatase (PPase) (New England Biolabs, Frankfurt am Main, Germany).
- RiboLock RNase inhibitor (RiboLock) (Thermo Scientific).
- TB2 (5x): 200 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM spermidine, 50 mM DTT, 30 mM $MgCl_2$, 50 mM NaCl, dissolve the above components with diethylpyrocarbonate (DEPC)-treated H_2O, then adjust pH to 7.8 with HCl. Divide the buffer into 1 mL aliquots and store at -20˚C.
- rNTP mix: mix 36 µL of 100 mM rATP (Promega, Mannheim, Germany), 36 µL of 100 mM rCTP (Promega), 36 µL of 100 mM rGTP (Promega) and 36 µL of 100 mM rUTP (Promega) with 256 µL DEPC-treated H_2O, store at -20˚C.
- RNA Clean & Concentrator™-5 Kit (ZYMO Research, Freiburg, Germany).
- DNaseI (RNase-free) (Thermo Scientific).
- NanoDrop 2000 Spectrophotometer (Thermo Scientific).
- The RNAs of PET esterase candidates can be synthesized by self-made RNA polymerase EM1 RNAP with previously produced DNA templates (*see* section 3.1) in cell-free system.
- Pipette the reaction as indicated in **Table 3**:

Table 3. *in vitro* transcription reaction mix.

Components	Amount
TB2 (5x)	10 µL
rNTPs mix	10 µL
PPase 2 U/µL	2.5 µL

Ribolock 40 U/µL	1.25 µL
EM1 RNAP	5 µg
DNA templates	1 µg
RNase-free water	Add to 50 µL

Incubate the reaction mixture at 37°C for 2 hours.

Digest the DNA template with 2 µL DNase I (1 U/µL) per reaction, incubate the reaction mixture at 37°C for additional 15 min.

Purify the produced RNA with RNA Clean & Concentrator™-5 kit according to the manufacturer's instruction and elute RNA with 17 µL DEPC-treated H_2O.

Take 2 µL RNA for quantification in NanoDrop 2000 Spectrophotometer.

The amount of RNA produced via *in vitro* transcription can be alternatively checked with Agilent RNA 6000 Pico chip in Agilent 2100 Bioanalyzer (Agilent Technologies) or with Qubit™ RNA HS Assay Kit in the Qubit ® 3.0 fluorometer (ThermoFisher Scientific, Darmstadt, Germany). The size of RNA can be verified either with Bioanalyzer or with gel electrophoresis. In our routine experiments, good production of RNA from *in vitro* transcription is around 3 ~ 5 µg/µL and 15 µL RNA is added to one *in vitro* translation reaction.

3.3. Preparation of cell extract

List of required materials:

- LB medium: dissolve 10 g NaCl, 10 g tryptone and 5 g yeast extract in 1 L H_2O, autoclave.
- *E. coli* BL21-CodonPlus (DE3)-RIL (Agilent Technologies, Waldbronn, Germany).
- Buffer A: 10 mM Tris, 14 mM magnesium acetate, 60 mM monopotassium glutamate, dissolve the above components with DEPC-treated H_2O, then adjust pH to 8.2 with acetate, store at 4˚C. Add dithiothreitol (DTT) freshly to final concentration of 2 mM.
- Sonicator for cell lysis.

Cell extract provides many factors for translation and protein folding, therefore, it is very important for the translation efficiency. Several bacterial strains have been tested in our lab. Finally, *E. coli* BL21-CodonPlus (DE3)-RIL was chosen for our routine *in vitro* translation, with which good amounts of target proteins were produced.

Grow *E. coli* BL21-CodonPlus (DE3)-RIL cells overnight in 25 mL LB medium without antibiotics in a 100 mL-flask at 37˚C with continuous shaking at 150 rpm.

On the second day, inoculate two percent of the starter culture into 300 mL fresh LB medium in a 1 L-flask (1:50 ratio) and grow them at 37˚C on a shaker with a speed of 150 rpm until its mid-exponential growth phase (OD_{600} ≈ 2.0, normally, it takes about 4 ~ 5 hours to reach this cell density).

Harvest cells by centrifugation with a speed of 6,500 *x g* at 4˚C for 15 min.

Wash the cells three times with pre-cooled Buffer A supplied with 2 mM DTT just before use.

After final wash and centrifugation, weigh the pelleted wet cells, flash freeze them in liquid nitrogen, and store at -70˚C overnight. Cell pellets should not be stored for more than three days.

On the third day, thaw cells on ice and suspend them in 1 mL of Buffer A (containing 2 mM DTT) per 1 g of wet cell mass.

Keep the suspension on the ice-water bath and break the cells by means of ultrasound (sonication): 6x 1 min with 1 min interval, output ctrl 0.5, duty cycle 50%.

Remove cell debris and other insoluble components by centrifugation at 4˚C with a speed of 16,000 *x g* twice, each time for 20 min. Keep the final supernatant.

Divide the supernatant (cell extract) in aliquots of 50 ~ 60 µL, flash freeze them in liquid nitrogen and store at -70˚C until use.

Buffers and wares should be pre-cooled and handling should be on ice all the time. To avoid any source of RNase contamination, use gloves all the time during extract preparation, DEPC-treated H_2O for buffer preparation (possibly autoclaved afterwards), and autoclaved wares. The nuclease-free and safe-locked tubes should be used for aliquots of cell extract.

3.4. *In vitro* Translation

List of required materials:

- ivTT buffer (10x): 340 µg/mL folinic acid, 1.3 M potassium glutamate, 100 mM ammonium acetate, 120 mM magnesium glutamate, 1.5 mM spermidine, 10 mM putrescine, 40 mM sodium oxalate and 2.7 mM coenzyme A. Dissolve all above components in DEPC-treated H_2O. Divide the buffer into 1 mL aliquots and store at -20˚C.
- PEP (10x): dissolve 0.15 g phosphoenolpyruvate trisodium salt heptahydrate (MW = 360.09 g/mol) in 1.26 mL DEPC-treated H2O. Divide the solution into 0.1 mL aliquots and store at -20˚C.
- tRNA mix: dissolve 0.0102 g tRNA from *E. coli* MRE 600 (Roche Diagnostics, Mannheim, Germany) in 1 mL DEPC-treated H_2O. Divide the solution into 0.1 mL aliquots and store at -20˚C.
- NAP/ATP solution: dissolve 0.022 g nicotinamide adenine dinucleotide (NAD) and 0.0193 g adenosine 5'-triphosphate disodium salt (ATP) in 10 mL DEPC-treated H_2O. Divide the solution into 0.1 mL aliquots and store at -20˚C.
- aa-mix (amino acids mixture, 20 mM each): to prepare 5 mL aa-mix (**Table 4**), dissolve 19 essential amino acids (except tyrosine) as listed in the following table in 4 mL DEPC-treated H_2O. Dissolve 0.0906 g tyrosine (Y, MW = 181.19 g/mol) in 1 mL of 5 M KOH to get 500 mM tyrosine stock solution, then add 0.2 mL stock solution to aa-mix. Fill DEPC-treated H_2O to 5 mL, and then the final concentration of each amino acid is 20 mM. Divide the solution into 0.5 mL aliquots and store at -20˚C.

Table 4. aa-mix (amino acids mixture, 20 mM each)

L-amino acid	MW (g/mol)	Amount for 20 mM in 5 mL solution (g)
A (alanine)	89.09	0.0089
C (cystein)	121.16	0.0121
D (aspartic acid)	133.1	0.0133
E (glutamate) (sodium salt)	187.14	0.0187
F (phenylalanine)	165.19	0.0165
G (glycine)	75.07	0.0075
H (histidine)	155.16	0.0155
I (isoleucine)	131.17	0.0131
K (lysine)	146.19	0.0146

L (leucine)	131.18	0.0131
M (methionine)	149.21	0.0149
N (asparagine) (monohydrate)	150.14	0.0150
P (proline)	115.13	0.0115
Q (glutamine)	146.15	0.0146
R (arginine)	174.2	0.0174
S (serine)	105.09	0.0105
T (threonine)	119.12	0.0119
V (valine)	117.15	0.0117
W (tryptophan)	204.23	0.0204

In this step, the candidate protein will be produced with tRNAs and the translation machineries from above prepared cell extract in a cell-free system using *in vitro* transcribed RNA as template.

1. Pipette the reaction as indicated in **Table 5**:

Table 5. *In vitro* translation reaction mixture

Components	Volume (µL)	
	Sample	Negative control
ivTT buffer (10x)	6	6
PEP (10x)	6	6
NAD/ATP (10x)	6	6
tRNA mix	1	1
aa-mix (20 mM)	6	6
Cell extract	13.8	13.8
Ribolock 40 U/µL	1.8	1.8
RNA (produced from ivTx)	15	-
RNase-free water	Add to 60 µL	

2. Incubate the reaction mixture at 37°C for 4 hours.

Check the activity of *in vitro* produced protein with appropriate assay. In our case, the DNA sequences were synthesized in vector pET-21a(+) and amplified with primers binding to upstream and downstream of MCS, therefore, all PCR products contain the special sequence expressing 6x Histidine (His). Thus, the amount and the size of protein can be checked with Western-blot using anti-His antibody.
In the case of PET esterase, a BHET/TPA assay is applied.

3.5. Activity assay

List of required materials:

- BHET stock solution (1 M): add 0.254 g BHET (MW = 254.24 g/mol) in 1 mL dimethyl sulfoxide (DMSO), then heat the solution to 60°C and mix by agitation to dissolve BHET. Store at -20°C.
- TPA standards: for 1 M of TPA stock solution, dissolve 1.66 g TPA (MW = 166.13 g/mol) in 10 mL DMSO. Then dilute 1 M TPA stock with H_2O into 10 µM, 100 µM and 500 µM TPA standard solutions and with DMSO into 1 mM, 5 mM and 10 mM TPA standard solutions for HPLC. Store all solutions at -20°C.
- Phosphate-buffered saline (PBS, 10x) (Carl Roth, Karlsruhe, Germany).

- Acetonitrile (1% TFA): acidify acetonitrile with 1% volume of trifluoroacetic acid (TFA).
- HPLC mobile phase: The mobile phase for isocratic elution of the analytes is composed of acetonitrile and H_2O in a volume ratio of 20:80. H_2O is acidified with 0.1% (v/v) of TFA. Elution was carried out at a constant flow rate of 0.4 mL/min.
- HPLC analysis is carried out with an UltiMate™ 3000 UHPLC system (Thermo Scientific) using a Triart C18 column (YMC Europe GmbH, Dinslaken, Germany) with a column size of 2 x 100 mm, a particle size of 1.9 µm and a pore size of 120 Å. Absorption of the eluate was measured at 254 nm using a VWD-3400 detector (Thermo Scientific).
- Compass HyStar software package from Bruker (Billerica).

Most of PET esterases can break the ester bond of BHET to produce TPA and EG with MHET as intermediate product. Higher yield of TPA indicates higher activity of candidates for degradation of BHET. The amount of TPA can be quantified with ultrahigh performance liquid chromatography (UHPLC, with particles smaller than 2 µm, which is smaller than normal HPLC methods) with the elution time at 1.7 min.

1. Pipette the reaction as indicated in **Table 6**:

Table 6. Activity assay for *in vitro* expressed PETases using BHET as substrate

Components	Volume (µL)		
	Sample	Negative control (master mix for ivTT)	Buffer control (PBS)
ivTT products	60	-	-
master mix for ivTT (without RNA template)	-	60	-
BHET (1 M in DMSO)	1		
PBS (10x)	10		
H_2O	Add to 100 µL		

2. Incubate the reaction mixture at 28°C or 55°C for 4 days.
3. Pellet the insoluble components by centrifugation at a speed of 16,000 *x g* for 5 min. Take 50 µL supernatant for assay.
4. Add 200 µL acetonitrile (1% TFA) and incubate at room temperature for 10 min.
5. Remove the insoluble components by centrifugation at a speed of 16,000 *x g* for 3 min.
6. Transfer 200 µL supernatant into a HPLC vial and add 600 µL H_2O in each vial, mix well.

Repeat Step 4 - 7 for TPA standards. Analyze the breakdown products and the TPA standards in an UltiMate™ 3000 UHPLC system using a Triart C18 column with a dimension of 100 x 2.0 mm containing particles with 1.9 µm diameter. Perform isocratic elution using the HPLC running solution at a flowrate of 0.4 mL/min.Detect the product at 254 nm with a VWD-3400 detector. Quantify the peak area of TPA (normally at 1.7 min) with the Compass HyStar software package. And normalize the amount of TPA produced by PET esterase candidates to the TPA standards according to the area of TPA peaks.

4. Results of the *in vitro* expression and activity assays

4.1 *in vitro* transcription of PETase genes

For demonstration reasons, an example of a sequence mining process reported for the prediction of PETases was applied here. PETases genes were chosen because they are difficult to be found and only less than 40 such enzymes have been published (see PAZy database). Accordingly, 21 predicted PET-active candidate genes, together with well-characterized PET2 and PET30, were synthesized after codon optimization for *E. coli* system. Following the gene synthesis, a single PCR reaction was performed to introduce a specific promoter for EM1 RNAP (ORF45 promoter) upstream (5'-prime) of the target genes. PCR products were purified and transcribed into mRNA with EM1 RNAP. All 21 candidate genes and the two controls PET2 and PET30 were

successfully transcribed *in vitro* by EM1 RNAP (**Fig. 2**). All RNAs exhibited the correct size on an RNA gel figure annotated with Bioanalyzer and Agilent 2100 Expert software.

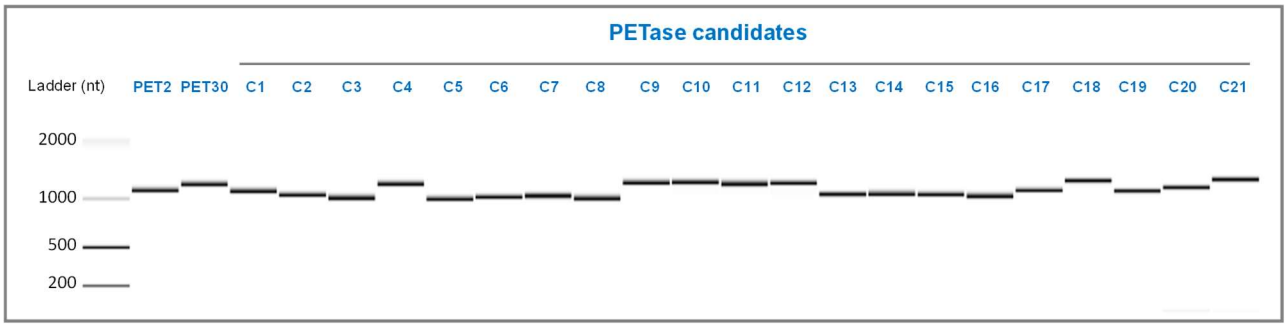

Figure 2. RNA gel showing mRNA of the *in vitro* transcribed putative PETase genes

Following the mRNA synthesis and quality control, the mRNAs were translated using ribosomal complexes from *E. coli* BL21-CodonPlus (DE3)-RIL cell extracts. After translation with *in vitro* produced mRNA as template in our self-established cell-free system, all produced candidates were applied to an activity assay using BHET as substrate. Notably, from the 23 translation assays, we observed 14 positive samples including the two positive controls. Thereby the amount of produced terephthalate (TPA) showed that these candidates exhibited different enzymatic activity concerning the degradation of BHET at the tested temperatures (**Fig. 3**). PET2 is active at both 28°C and 55°C, while PET30 is only active at a lower temperature. According to the yield of TPA, C20 shows the highest activity among screened 21 candidates on the degradation of BHET at 55°C and it is also quite active at 28°C, but much lower than 55°C; candidate C15 exhibits highest activity at 28°C and mild activity at 55°C. Candidate C9 - C13, and C17 are also active at 28°C. Besides, C2, C8, C18, and C19 did release a significant amount of mono-(2-hydroxyethyl) terephthalate (MHET) instead of TPA at 28°C (**Fig. 4**), adding another 4 active enzymes. Moreover, C9 - C12 produced significantly higher amount of MHET than the negative control, indicating that they are also active at 55°C. Thus, using this *in vitro* metagenomic screening method and employing EM1 RNAP novel enzyme candidates have been identified. These candidate genes have not been identified as BHET-active enzymes in previous work.

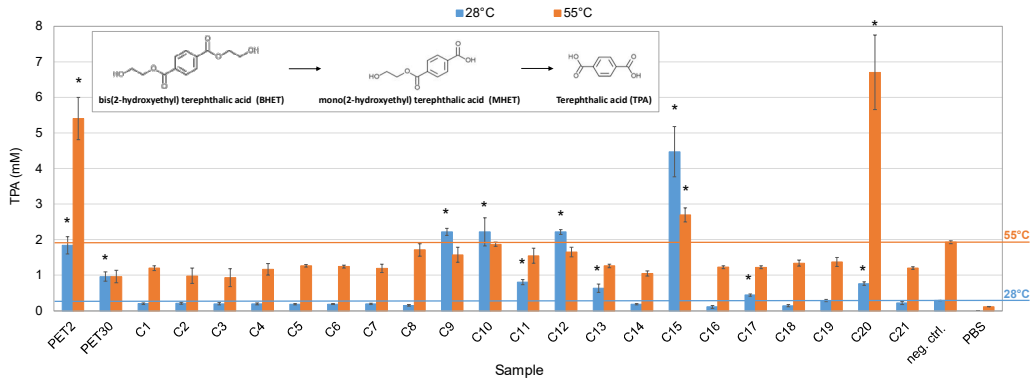

Figure 3. Amount of TPA released by enzymatic activity of *in vitro* expressed PETase candidates measured by UHPLC.

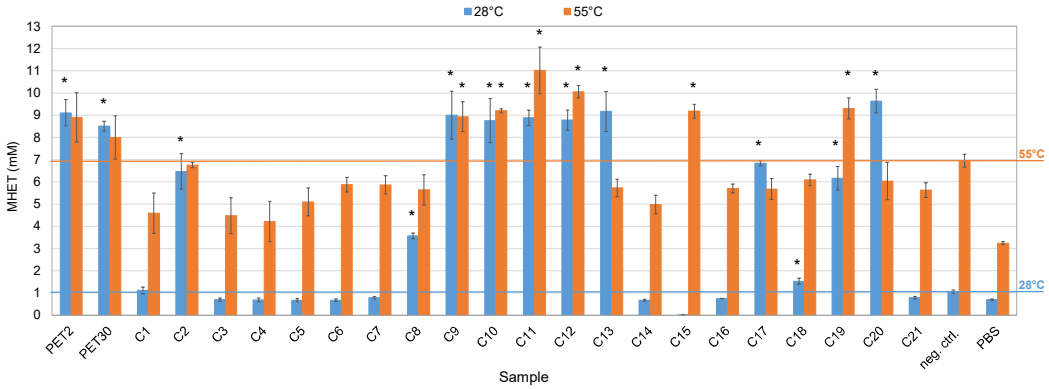

Figure 4. Amount of MHET released by enzymatic activity of *in vitro* expressed PETase candidates measured by UHPLC.

4.2 *in vitro* transcription of other proteins (cellulase, lipase, sfGFP)

Further, we wonder if other genes could be expressed based on an *in vitro* pipeline using EM1 RNAP. Therefore, the metagenome-derived cellulase CelA2, the lipase CalB, and the superfolder green fluorescent protein (sfGFP) were expressed using our cell-free protein expression system. In all cases, the amount of produced proteins was sufficient to perform the initial activity assay (**Fig. 5**). In the case of PETase (**Fig. 3 & 4**) and cellulase (**Fig. 5a**), the presence of translation mix or reaction components in the assay did not have significant impact on the yield. Therefore, the translation mix was directly applied to the activity assays. In the case of the expressed lipase (**Fig. 5b**), the *in vitro* expressed lipase was first immobilized to a 96-well nickel-coated microtiter plate to select only His-tagged lipase out of the translation extract, thereby removing the background lipolytic activities from the extract. Furthermore, sfGFP has been expressed with EM1 RNAP in cell-free polymersomes instead of normal reaction tubes. Such polymersomes with sfGFP enable the development of next-generation ultrahigh-throughput functional screening of metagenomes based on flow cytometry (**Fig. 5c**). All the above examples suggested that *in vitro* expressed proteins are active, and they exhibit detectable activity. Moreover, our *in vitro* expression system is a suitable tool to express a small number of proteins (20-50) in short time periods.

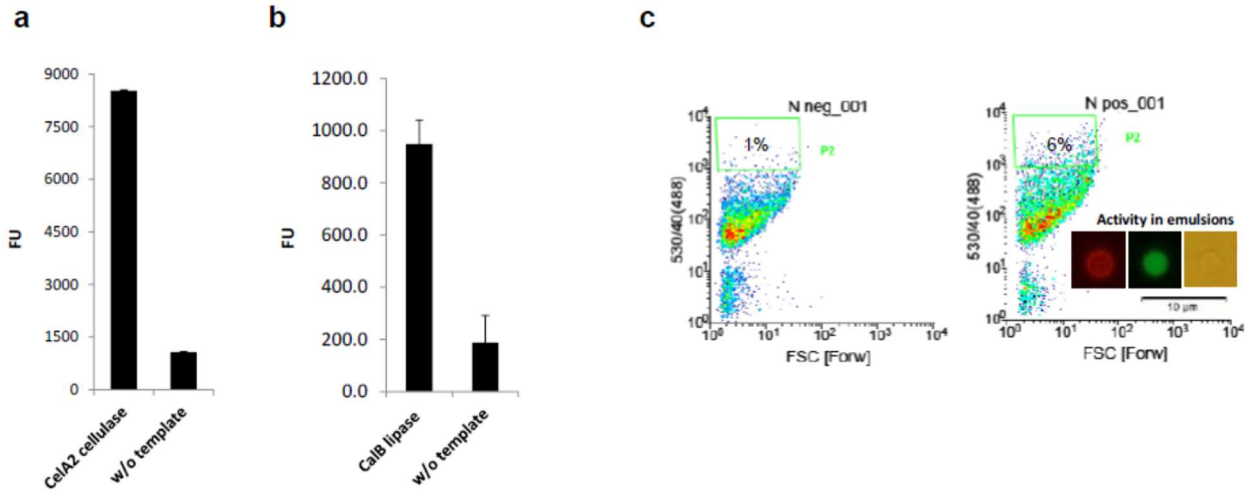

Figure 5. Example proteins produced with the EM1 RNAP *in vitro* expression system. Active *in vitro* expressed (a) cellulase CelA2 tested with 4-methylumbelliferyl β-D-cellobioside substrate and (b) lipase CalB tested with methylumbelliferyl caprylate after immobilization on a Ni^{2+}-coated microtiter plate. FU: fluorescence units. (c) *in vitro* expressed sfGFP was sorted by FACS with emulsion technology suitable for high throughput screening.

All cooperation partners are invited to plan experiments for this kind of activity screening and to send suitable plasmids, PCR products and if necessary substrates to UHAM for *in vitro* expression of the desired enzymes.