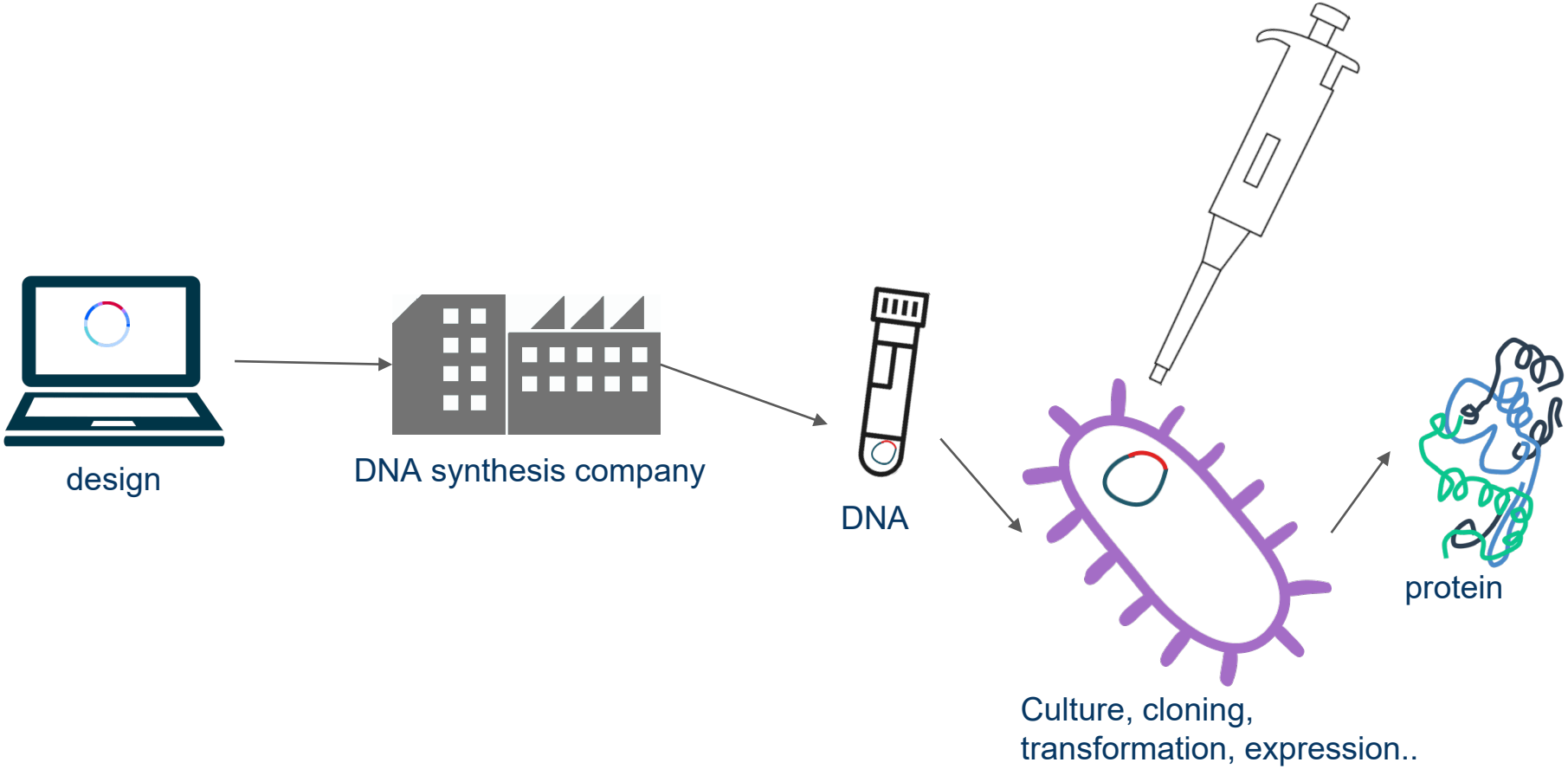
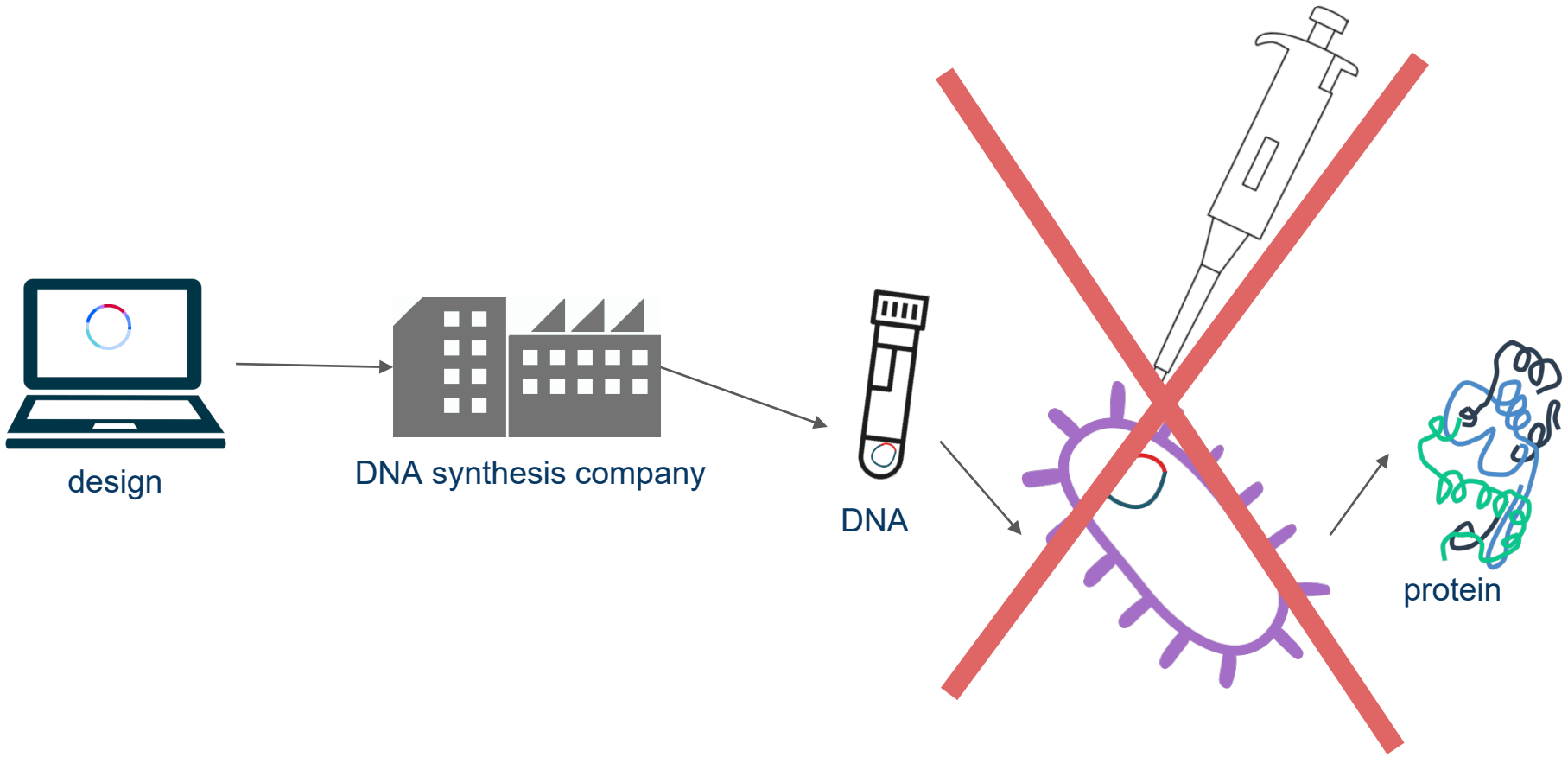
Introduction to Cell-Free systems

iGEM summer webinars

Alexis Casas
Imperial College London


design
DNA synthesis company
DNA
Culture, cloning,
transformation, expression..
protein


design
DNA synthesis company
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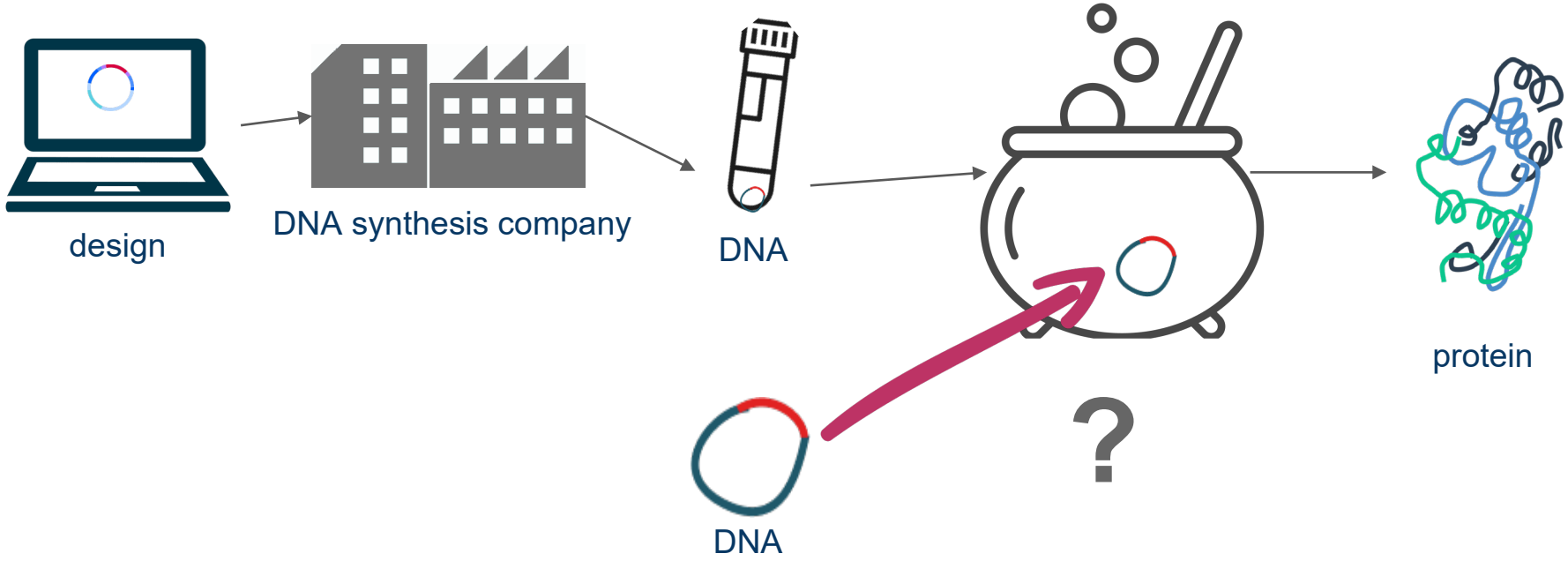
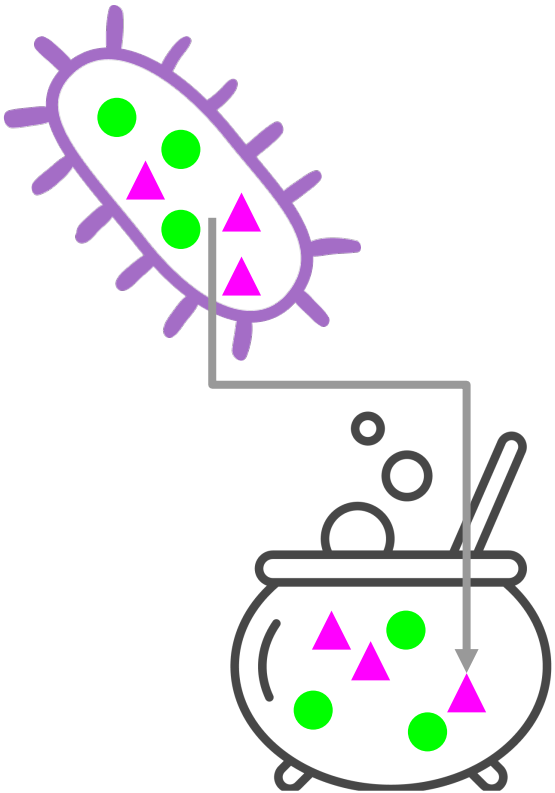
protein


design
DNA synthesis company
DNA
protein
DNA
?

What are Cell-Free systems ?

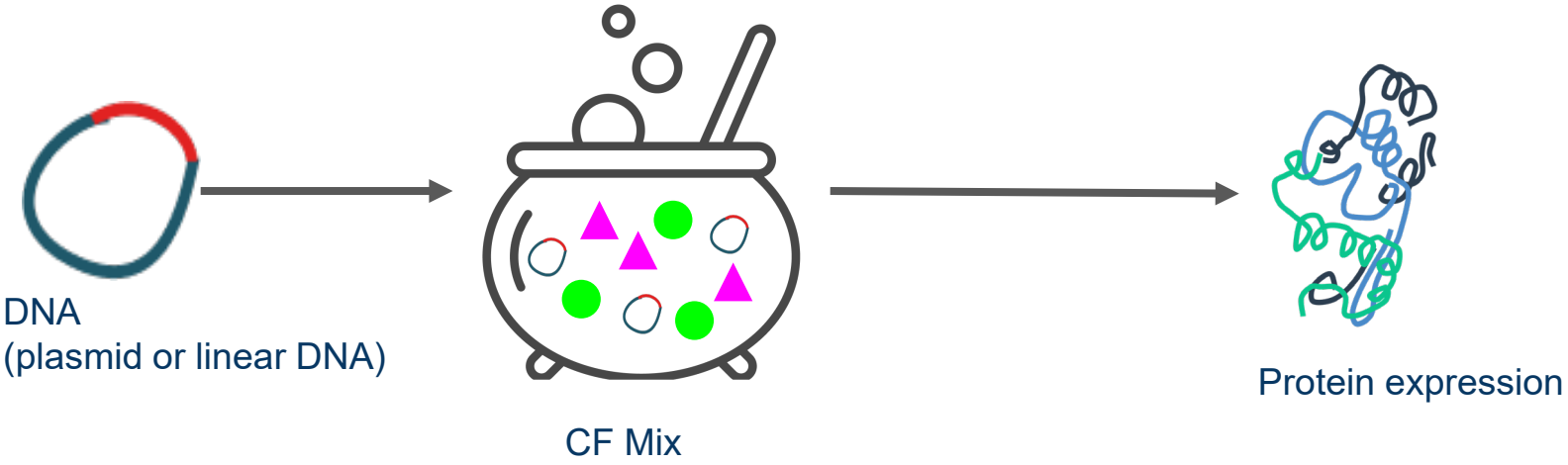
- Cell machinery without the cell walls
- Difference between *in-vivo* and *in-vitro* CF
  - Same machinery
  - Different constraints



- *In-vitro* TX-TL (Transcription and Translation)
- CFPS (Cell Free Protein Synthesis)
- CFPE (Cell Free Protein Expression)

What is TXTL ?

- TXTL = Transcription and Translation
- Cell-Free Mix + DNA → protein expression



Different Cell-Free systems

- Lysate based cell-free systems
  - Bacterial
    - *E. coli*
  - Eukaryotic
    - Yeast
    - Wheat germ
    - CHO cell lysate
    - Rabbit reticulocytes (immature red blood cells)
    - Insect cell lysate
    - Human cells (HeLa, HEK)

- PURE systems
  - Reconstituted cell-free protein synthesis system.
  - No black-box

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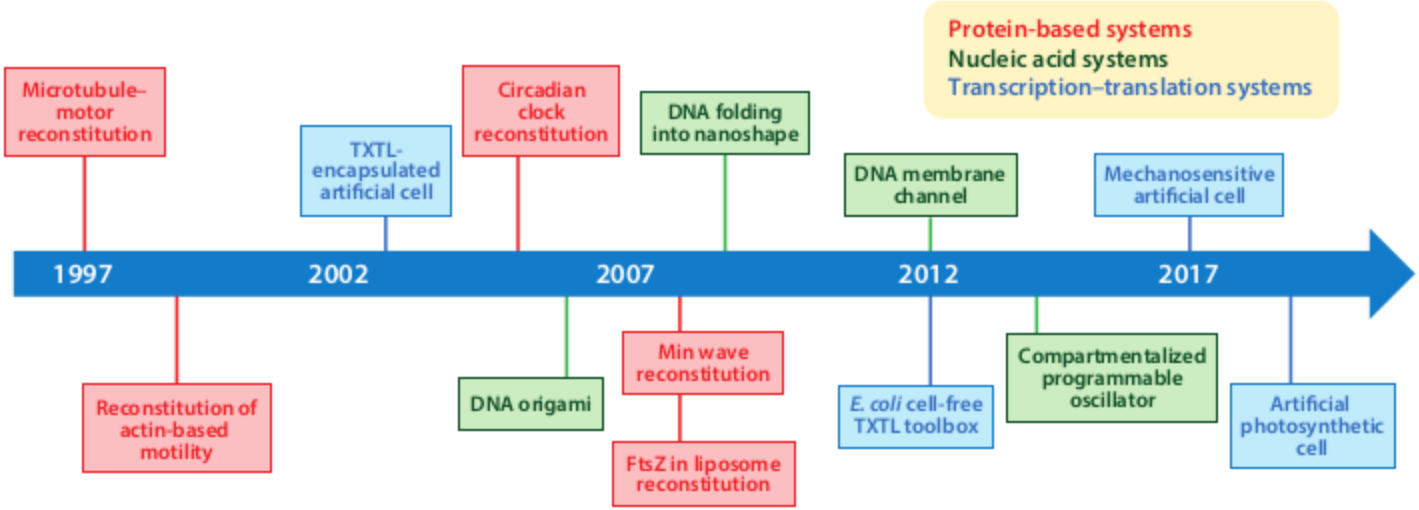
- PU
  -
  -

Third talk today

PURE System / One-Pot PURE - Barbora Lavickova

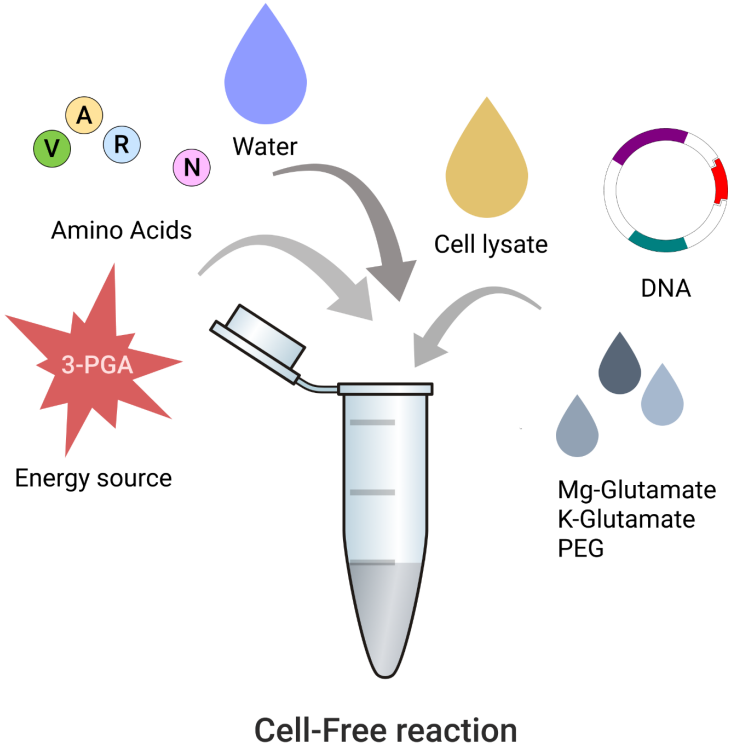
Cell-Free systems timeline



Noireaux, V. & Liu, A. P. The New Age of Cell-Free Biology. *Annu. Rev. Biomed. Eng.* **22**, 51–77 (2020).

How to make a Cell-Free TXTL mix ?

- Cell lysate (cell extract)
- Energy Mix
- NTPs
- Amino acids
- Salts
- Buffer
- ...



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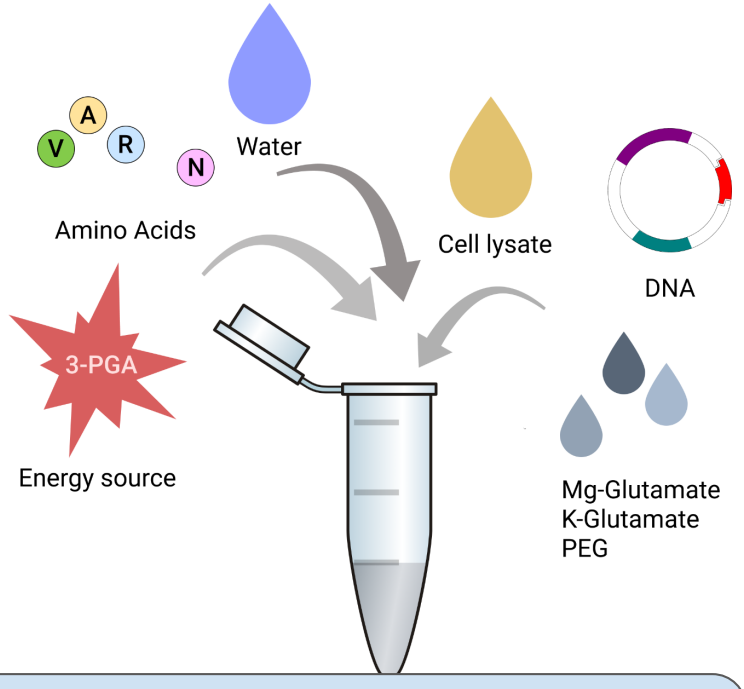


Next talk

Introduction to *E. coli* lysate systems - Zoe Swank

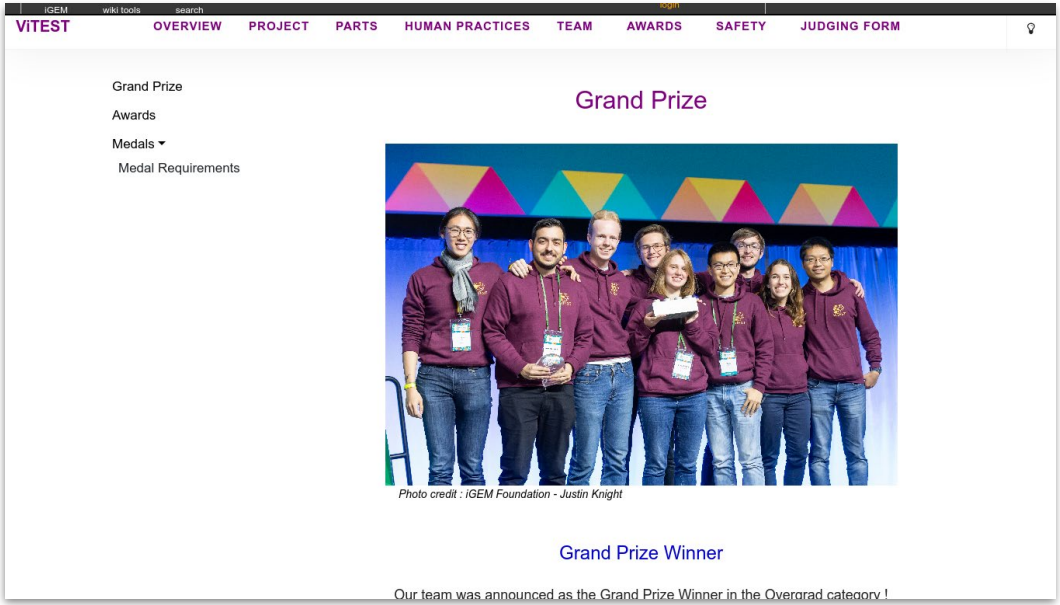
Cell-Free TXTL applications

- Protein expression
- Bio-sensors

Advantages of Cell-Free TXTL systems

- No transformation
- Protein expression system (toxicity for the cell etc..)
- Dry-freeze and rehydrated
- No biohazard - no GMO handling

Cell-Free TXTL systems in iGEM



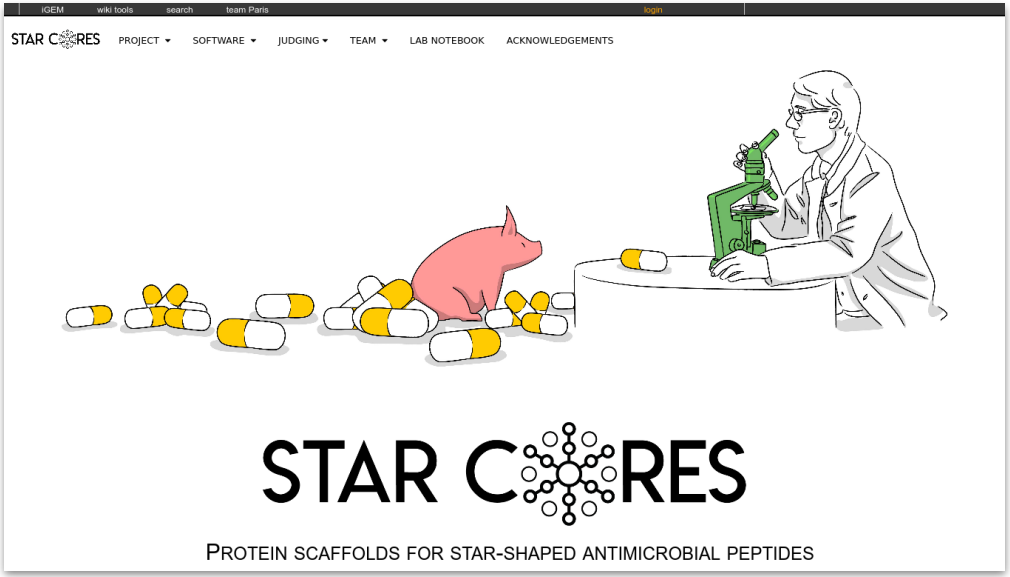
iGEM EPFL 2019

One-pot PURE
Paper-based CF detection system

Cell-Free TXTL systems in iGEM

iGEM Paris-Bettencourt 2018

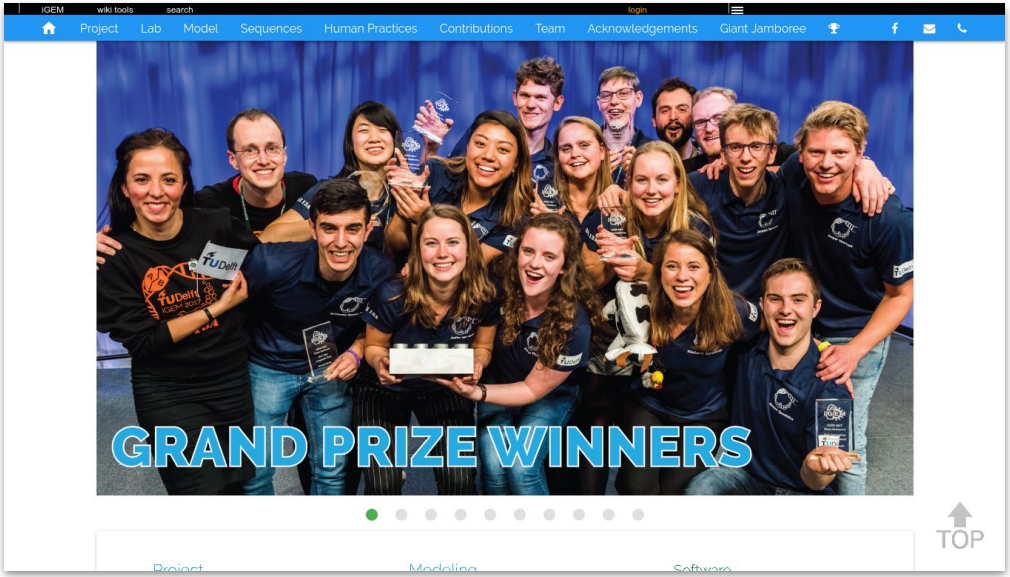
Antimicrobial peptides production in Cell-Free



Cell-Free TXTL systems in iGEM

iGEM TU Delft
2017

Detection of antibiotic resistance in bacteria that cause mastitis



Next talks on Cell-Free systems

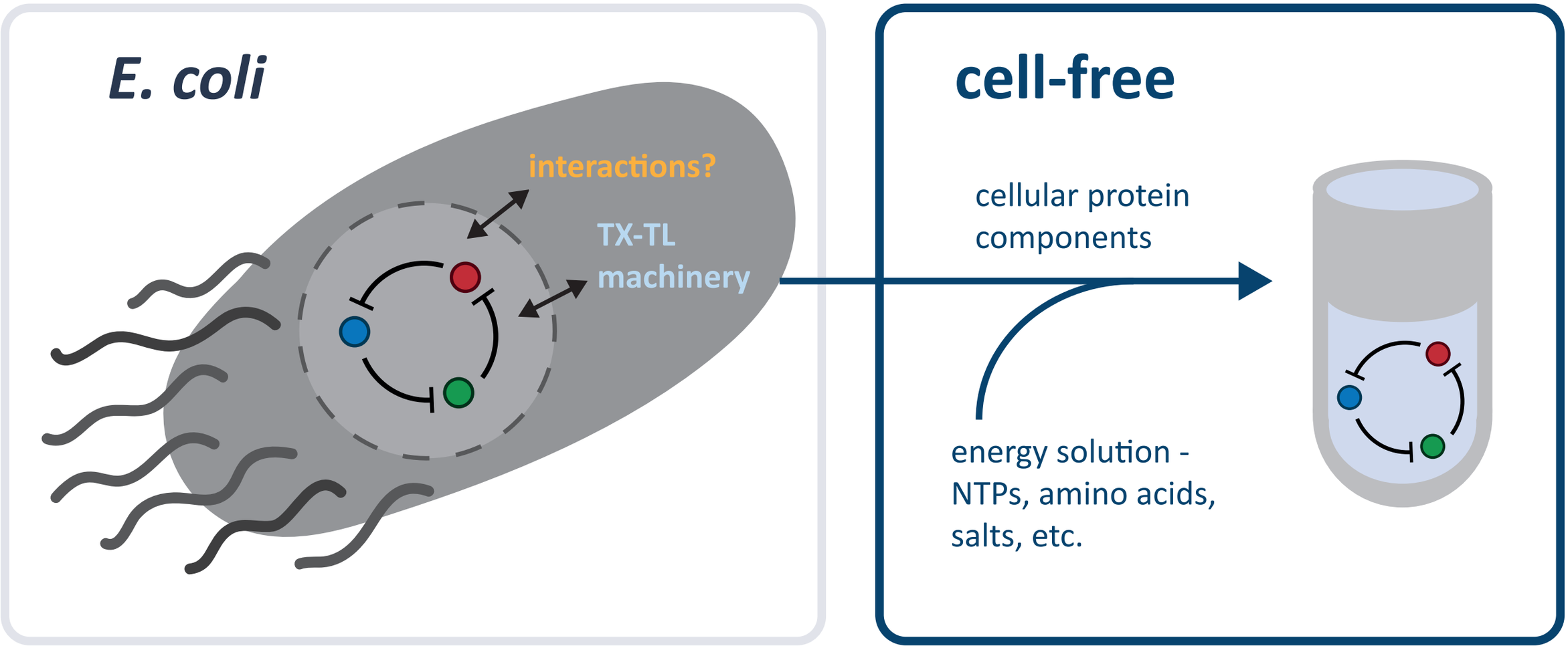
Introduction to *E. coli* lysate systems - Zoe Swank

PURE System / One-Pot PURE - Barbora Lavickova

Part 2
Intro to E. coli lysate systems

Zoe Swank
Prof. Sebastian Maerkl
EPFL

Benefits of transitioning to cell-free



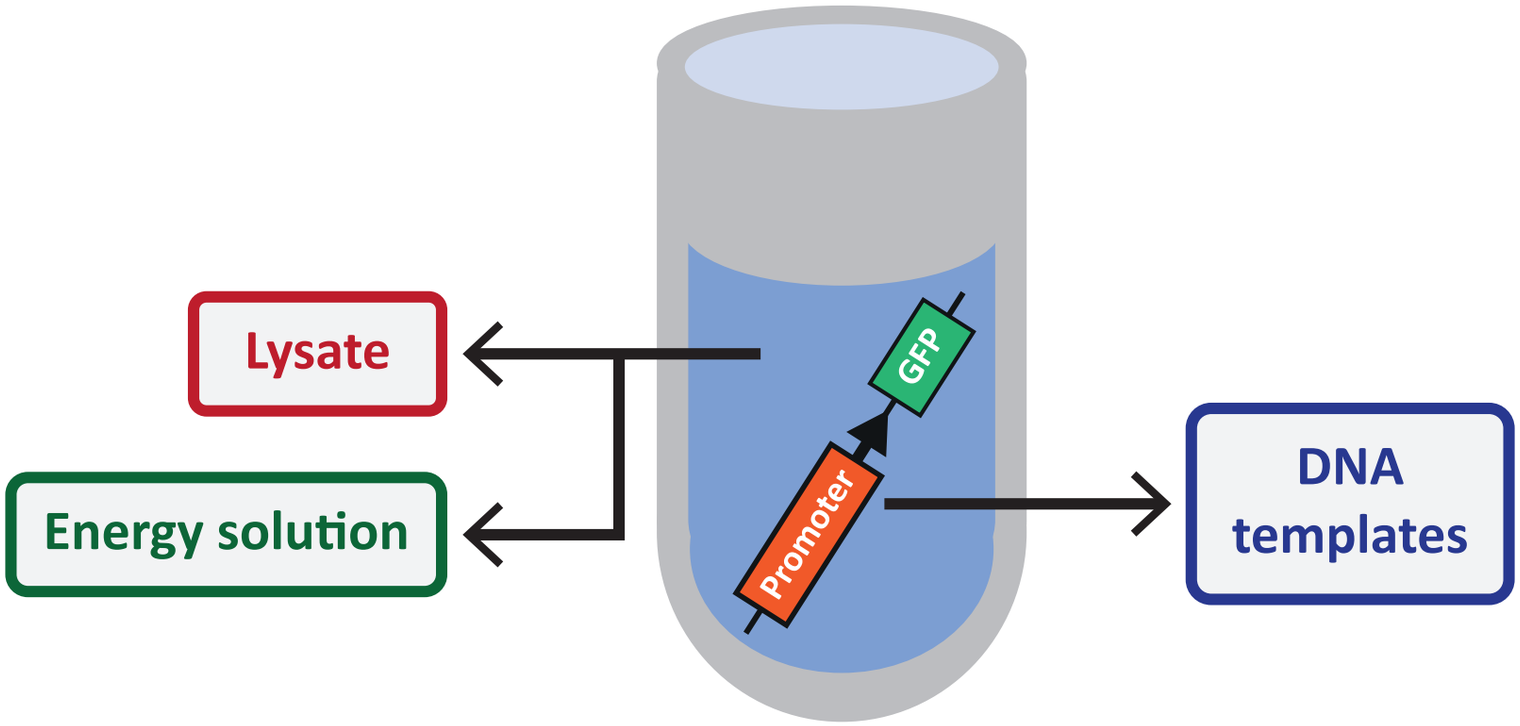
fluctuating environment

time-consuming molecular cloning

open, controllable system

linear DNA templates accelerate testing

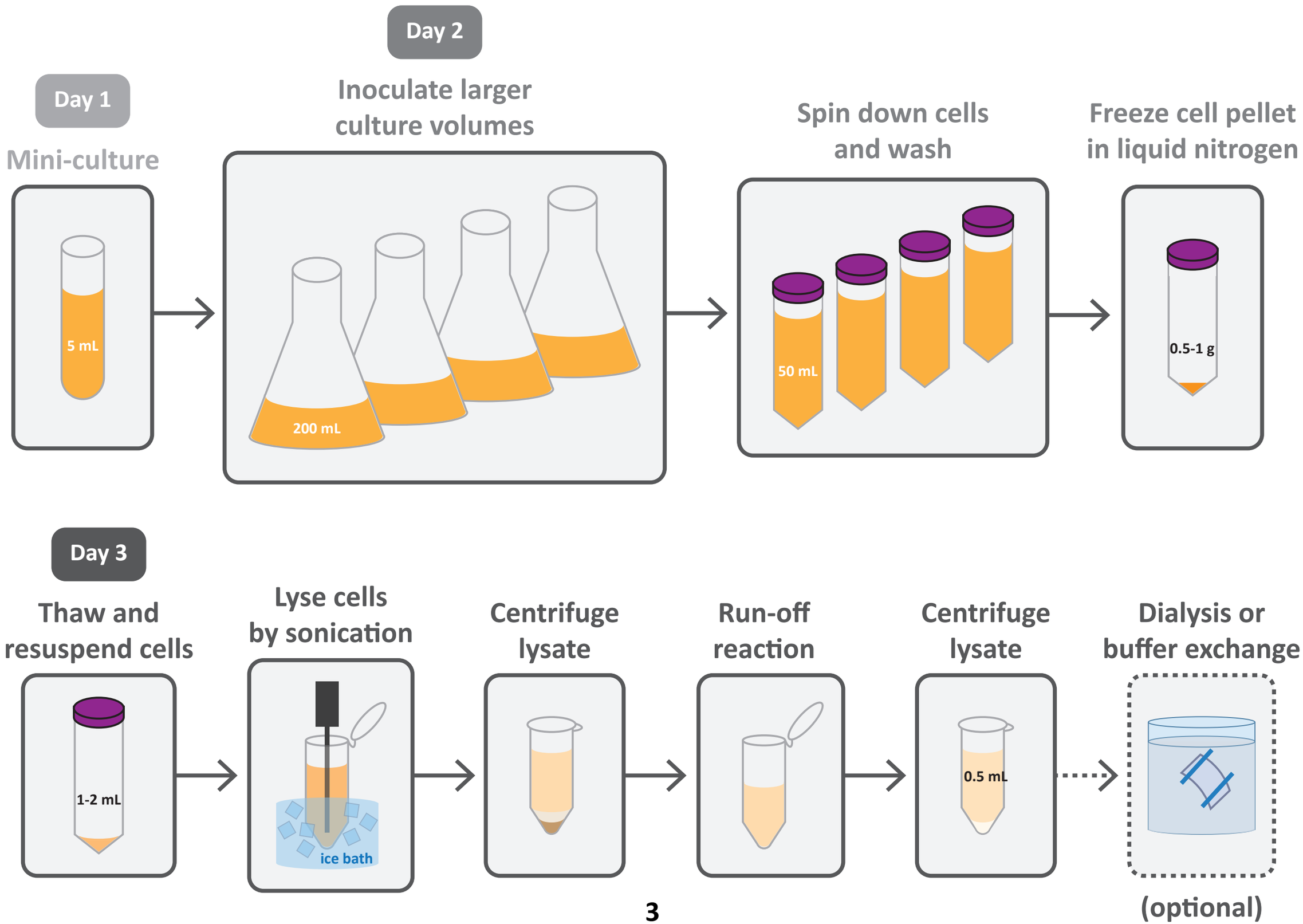
Overview of cell-free E. coli lysate systems



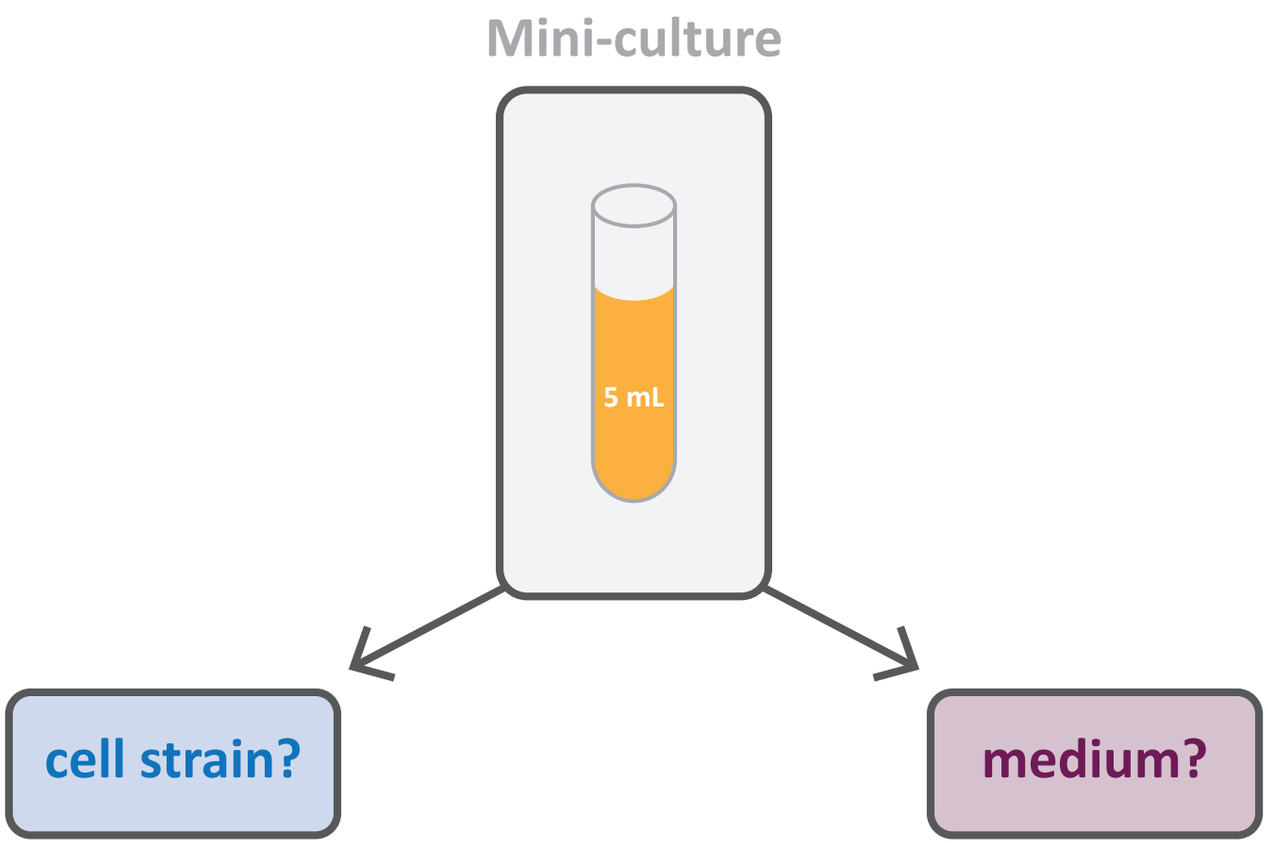
Resources:

- **Lysate**
  - Sun et al. 2013 *Jove*, Kwon et al. 2015 *Scientific Reports*, EPFL iGEM 2017 protocols
- **Energy solution**
  - Sun et al. 2013 *Jove*, Cai et al. 2015 *Biotechnology Progress*
- **DNA templates**
  - linear - Sun et al. 2014 *ACS Synth. Bio.*, Marshall et al. 2017 *Biotech. & Bioeng.*

Making lysate

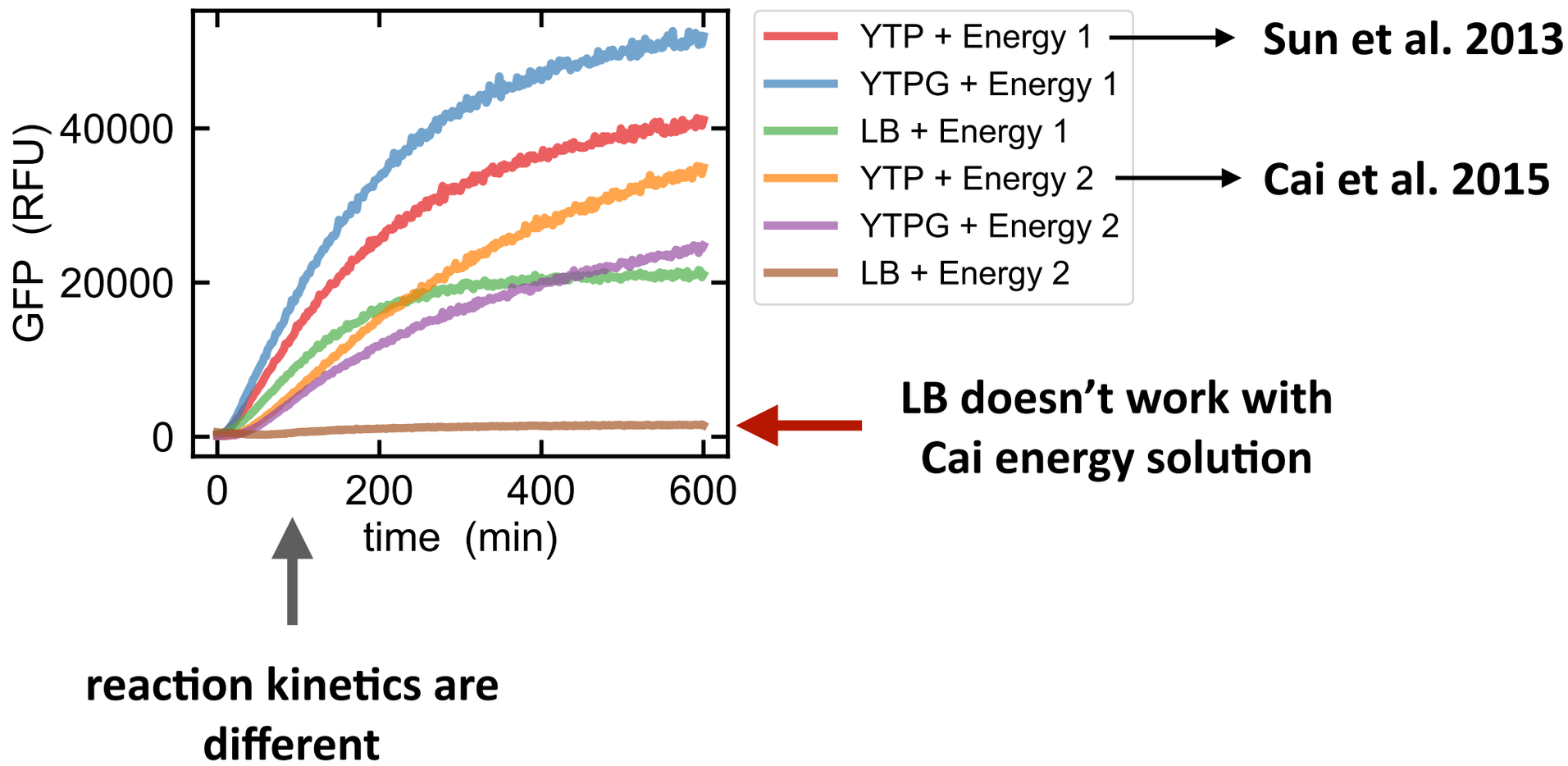


Things to consider - Day 1

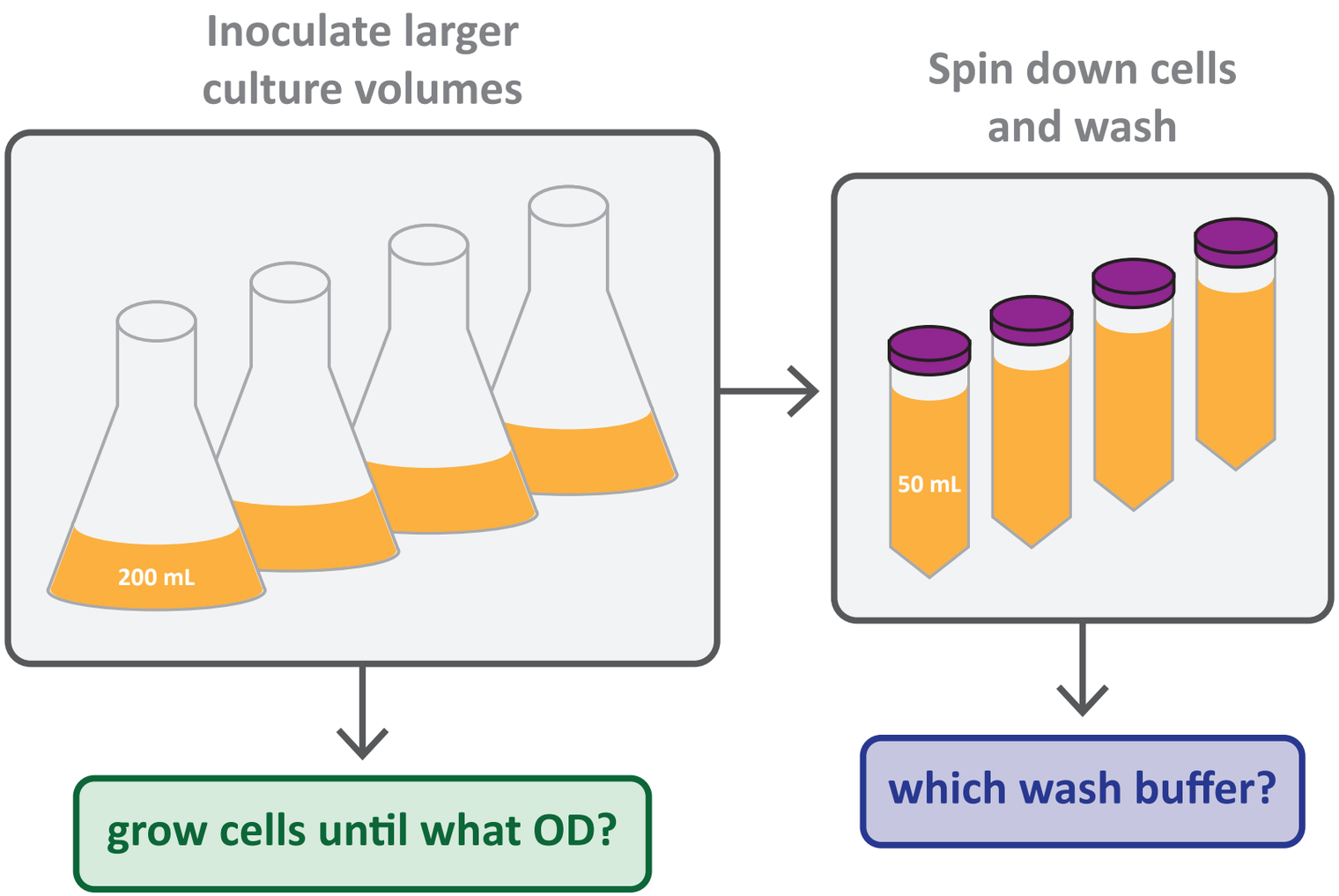


- Cell strain
  - BL21 (DE3) - many examples in literature, T7 RNAP
  - depends on the types of proteins you plan to express
- Medium
  - LB - works for energy solutions based on Sun et al. 2013
  - 2xYTP(G) - works for simplified and cheaper energy solution (Cai et al. 2015)

Example - energy solution

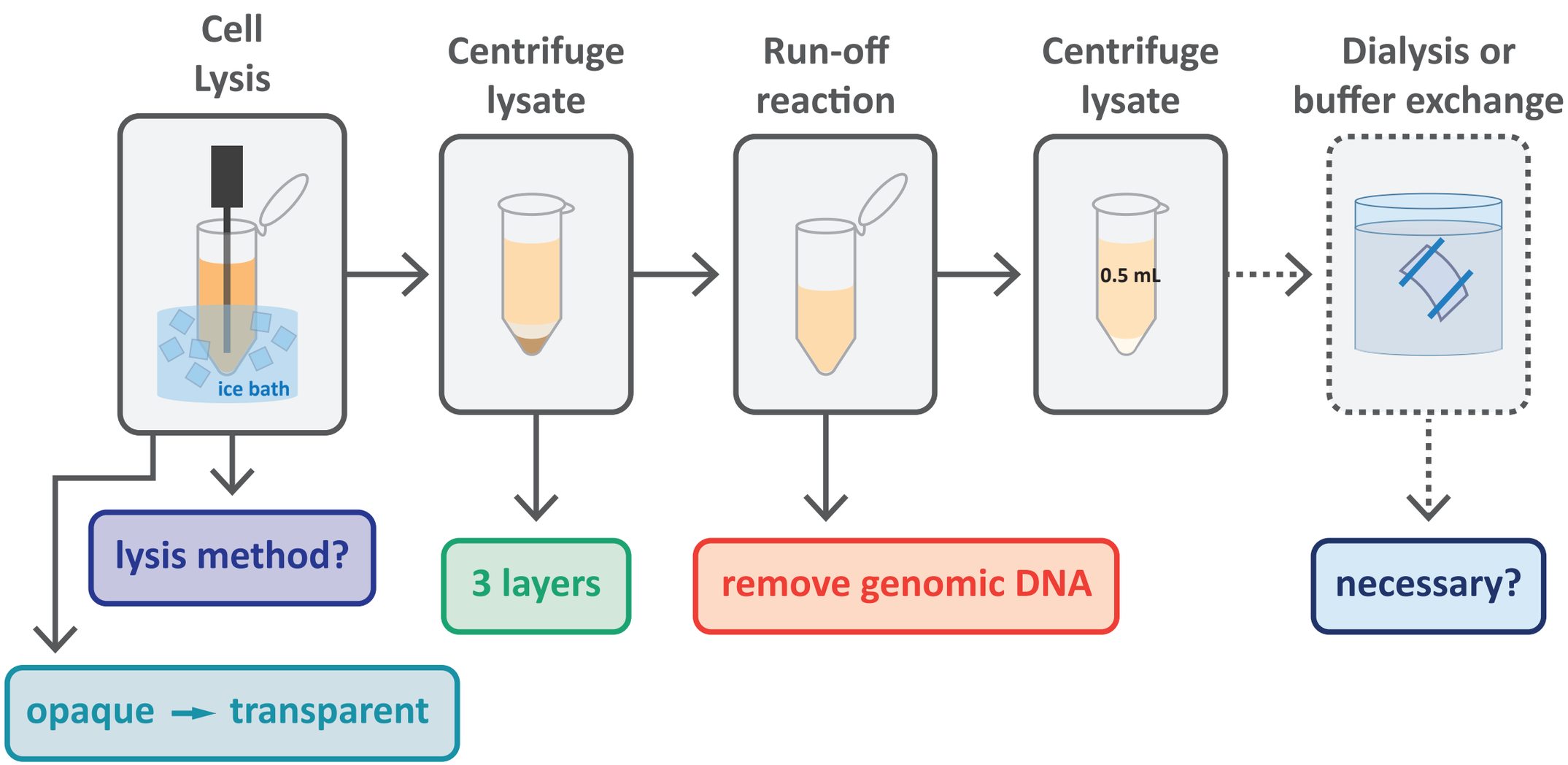


Things to consider - Day 2



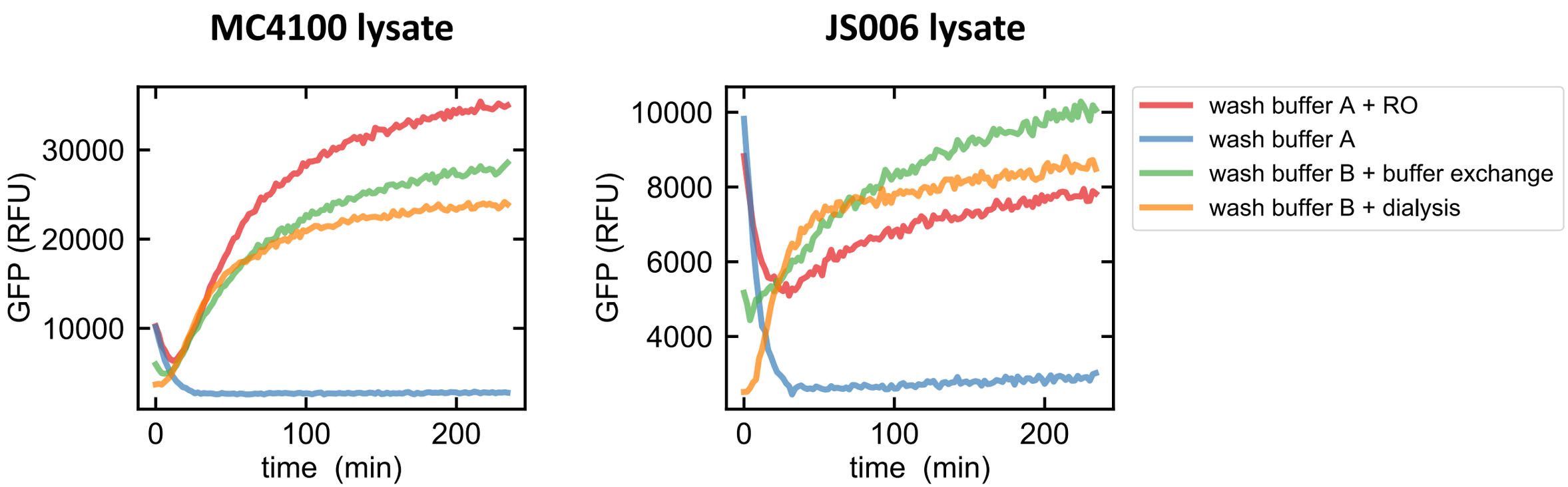
- OD
  - Sun et al. 2013 - exponential growth phase - OD = 1.5-2
  - depending on cell strain and preparation method a wide range of ODs can work
- Wash buffer
  - choose based on whether you opt for dialysis/buffer exchange

Things to consider - Day 3



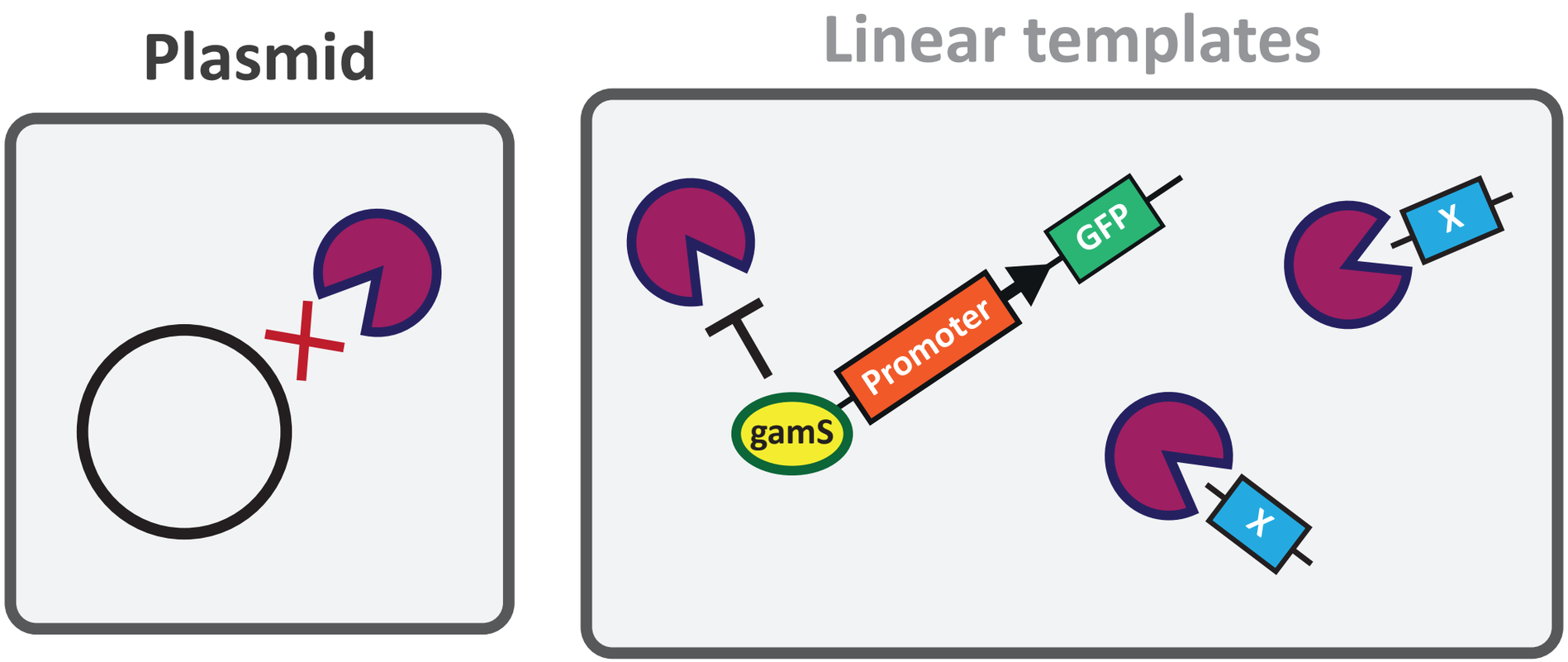
- Cell lysis
  - sonication (parameters are well described in Kwon et al.), french press, bead beating, etc.
- Dialysis or buffer exchange
  - can be important depending on cell strain
  - cellulose dialysis tubing, slide-a-lyzer mini dialysis units (small volumes), amino ultra centrifuge filters (buffer exchange, 0.5 mL)

Example - RO rxn, dialysis/buffer exchange



- **two lysates made from Lac- strains behave differently depending on the preparation methods**

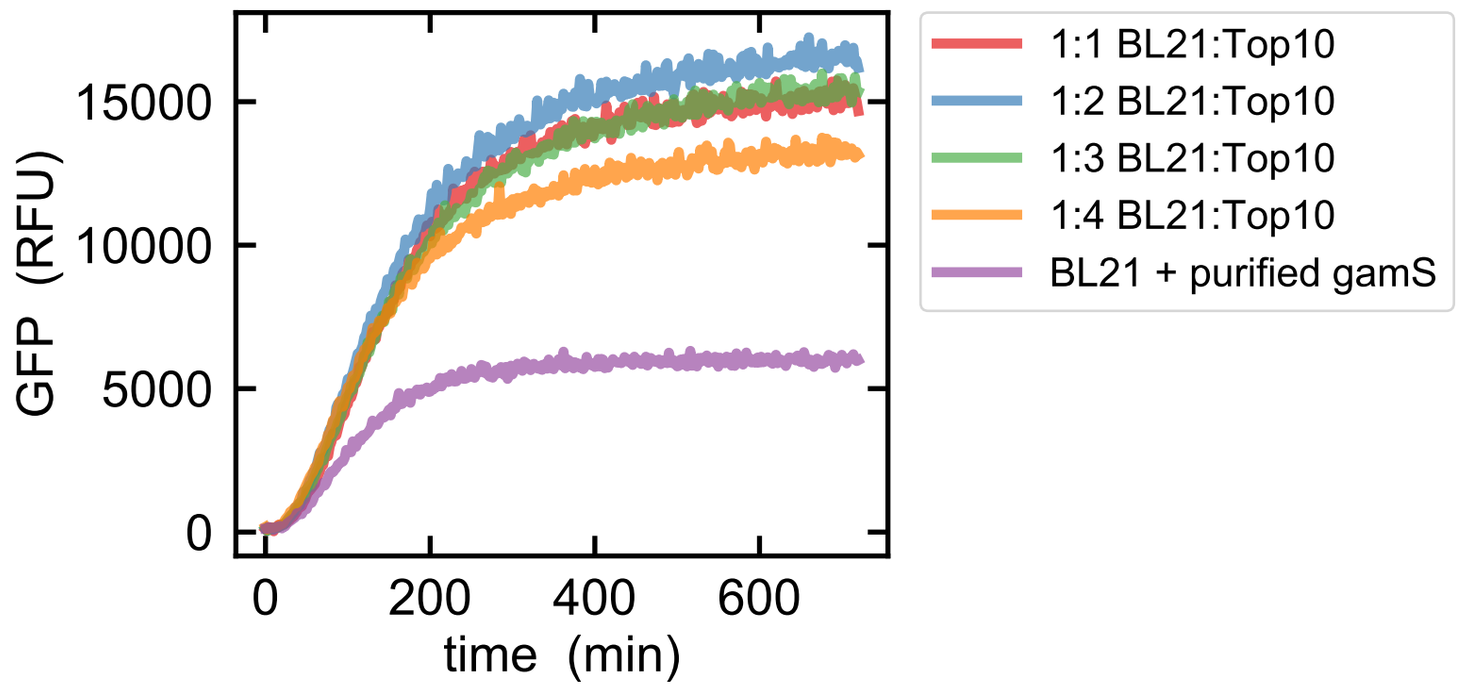
Testing lysate - DNA templates



- Plasmid DNA
- Linear DNA template
  - add either gamS (purified or lysate) or χ protection DNA

Example - gamS

- lysates can be mixed together
- saves time to purify a protein of interest

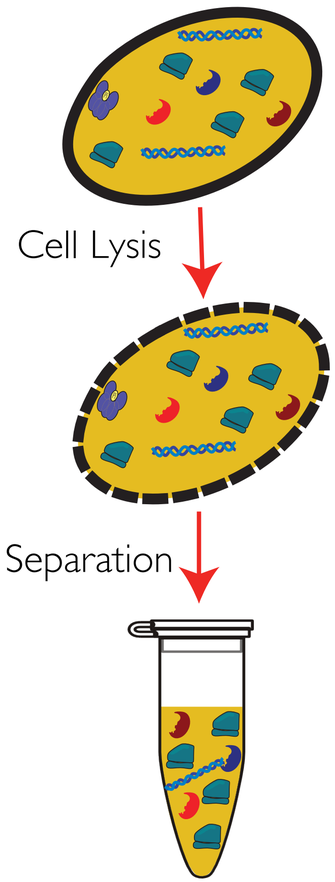


PURE system (recombinant TX-TL systems)

iGEM web seminars

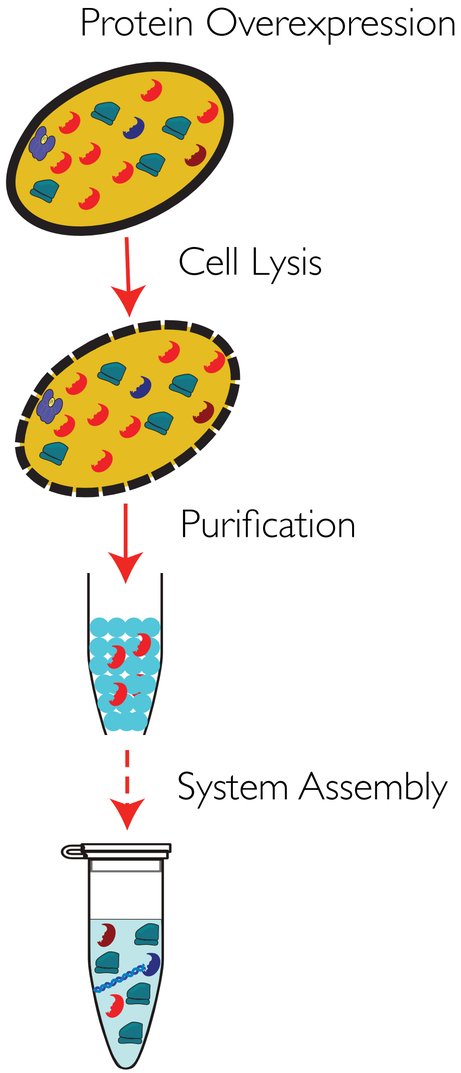
Barbora Lavickova
14.7.2020

Cell-free TX-TL systems



Proteins/Enzymes
LYSATE
RECOMBINANT

Lysate		PURE system
🙂	Prepation cost	☹️
🙂	Preparation time	☹️
🙂	Organism of origin	😐
🙂	Availability	😐
🙂	Reaction scaling	😐
☹️	Defined	🙂
☹️	Modularity	🙂
😐	Yield	😐
☹️	Yield per amount of proteins in the system	😐

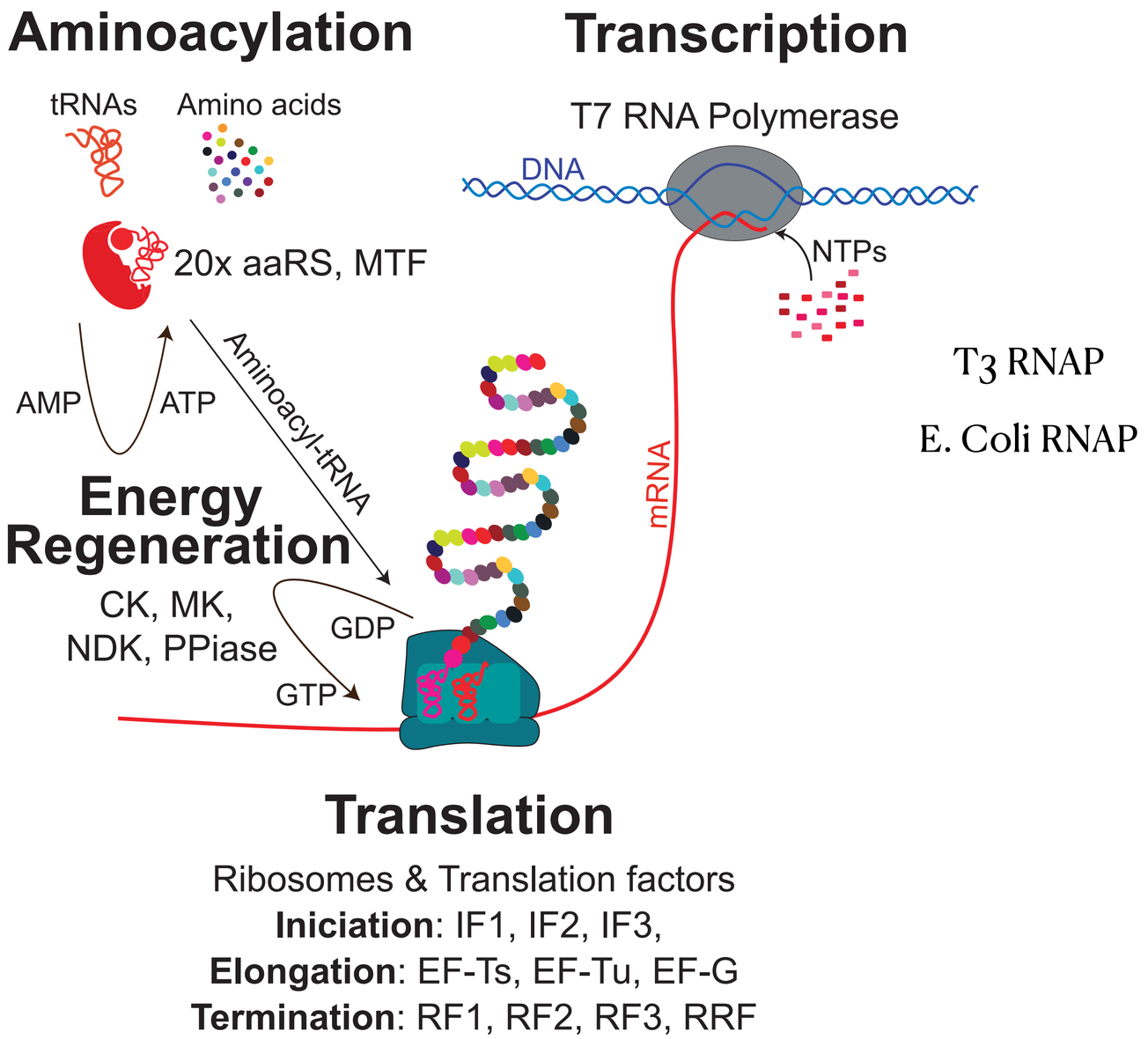


lysate TX-TL systems

protein synthesis using recombinant elements (PURE) - Shimizu, 2001

PURE system

- 36 proteins
- Ribosomes
- Small molecules (tRNA, AAs)
- Energy (CP, NTPs - ATP, GTP)
- Buffers - ions concentration (Mg^{2+})

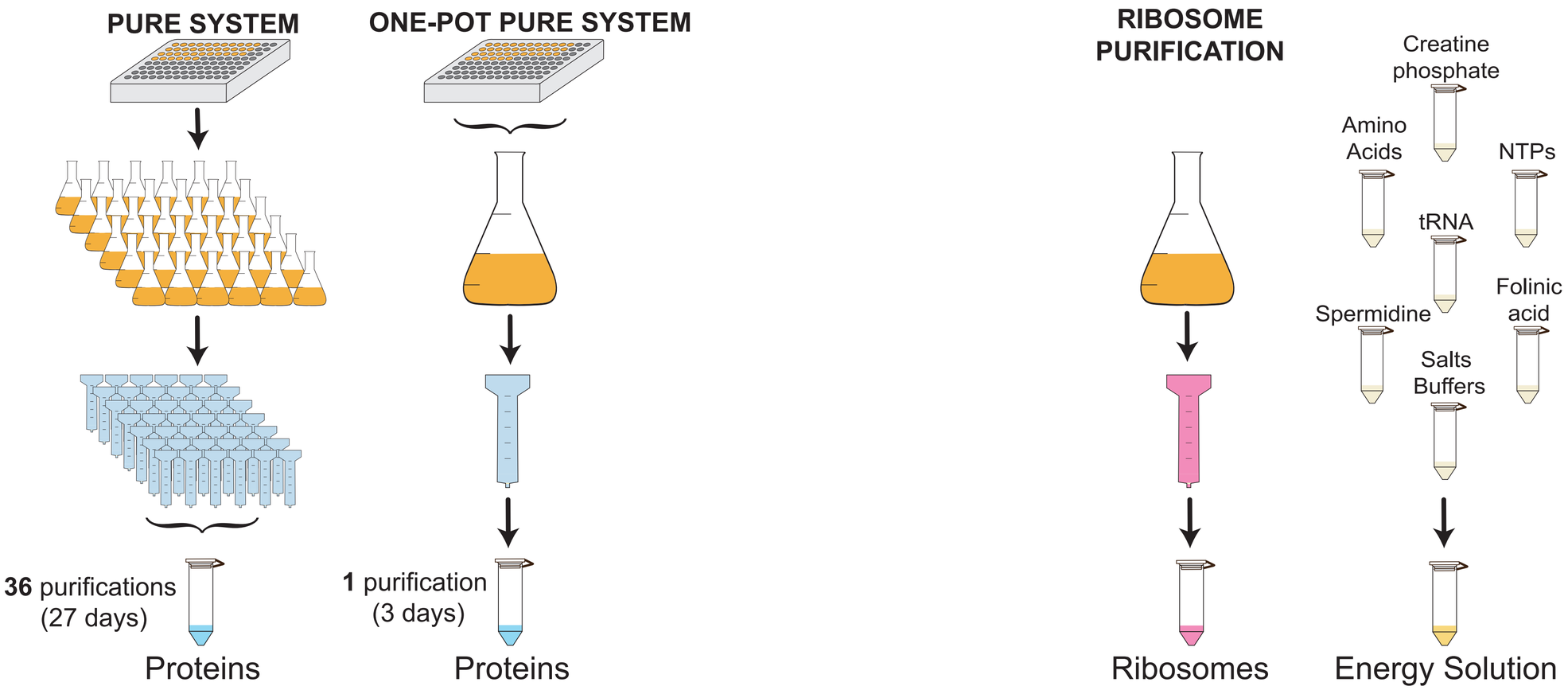


PURE / One-Pot PURE

Preparation

PURE / One-Pot PURE

Preparation

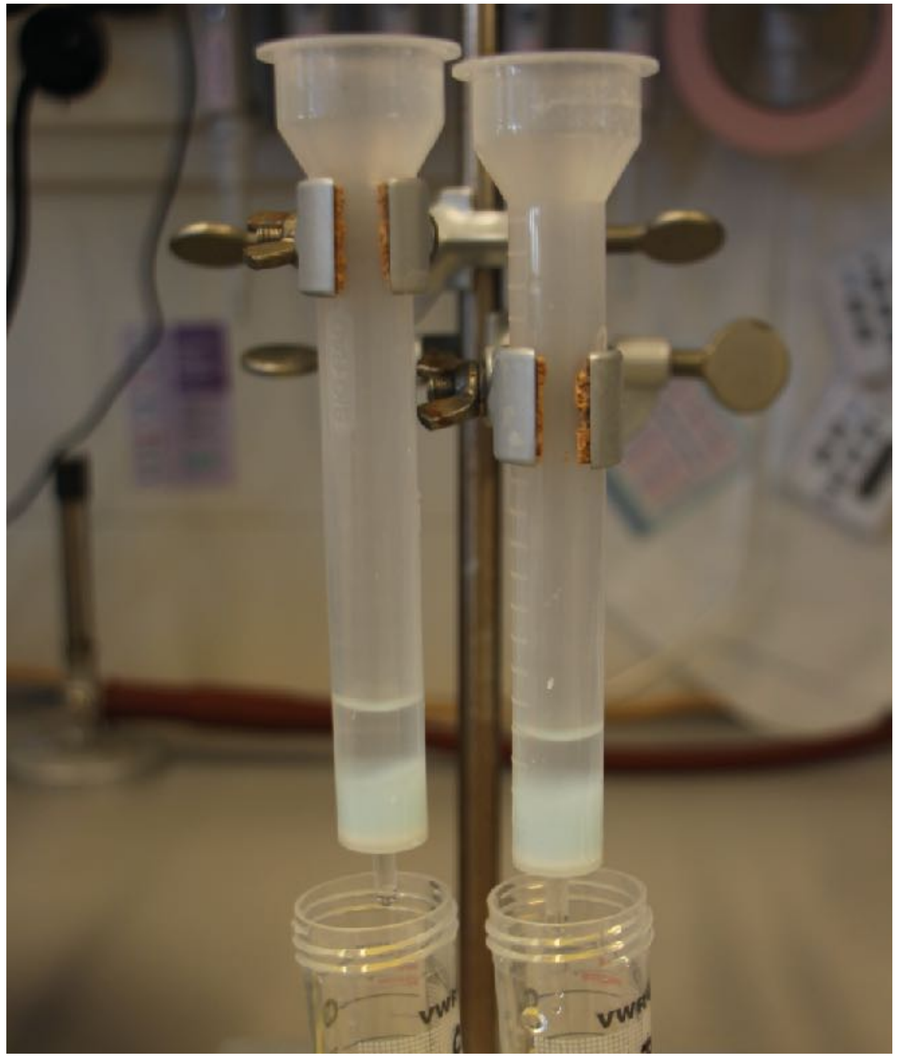


Protocols

- PURE (PURE Technology, Shimizu, 2010)
- One-pot PURE (A Simple, Robust, and Low-Cost Method To Produce the PURE Cell-Free System, Lavickova, 2019)
  - Supplementary Informations!!!!

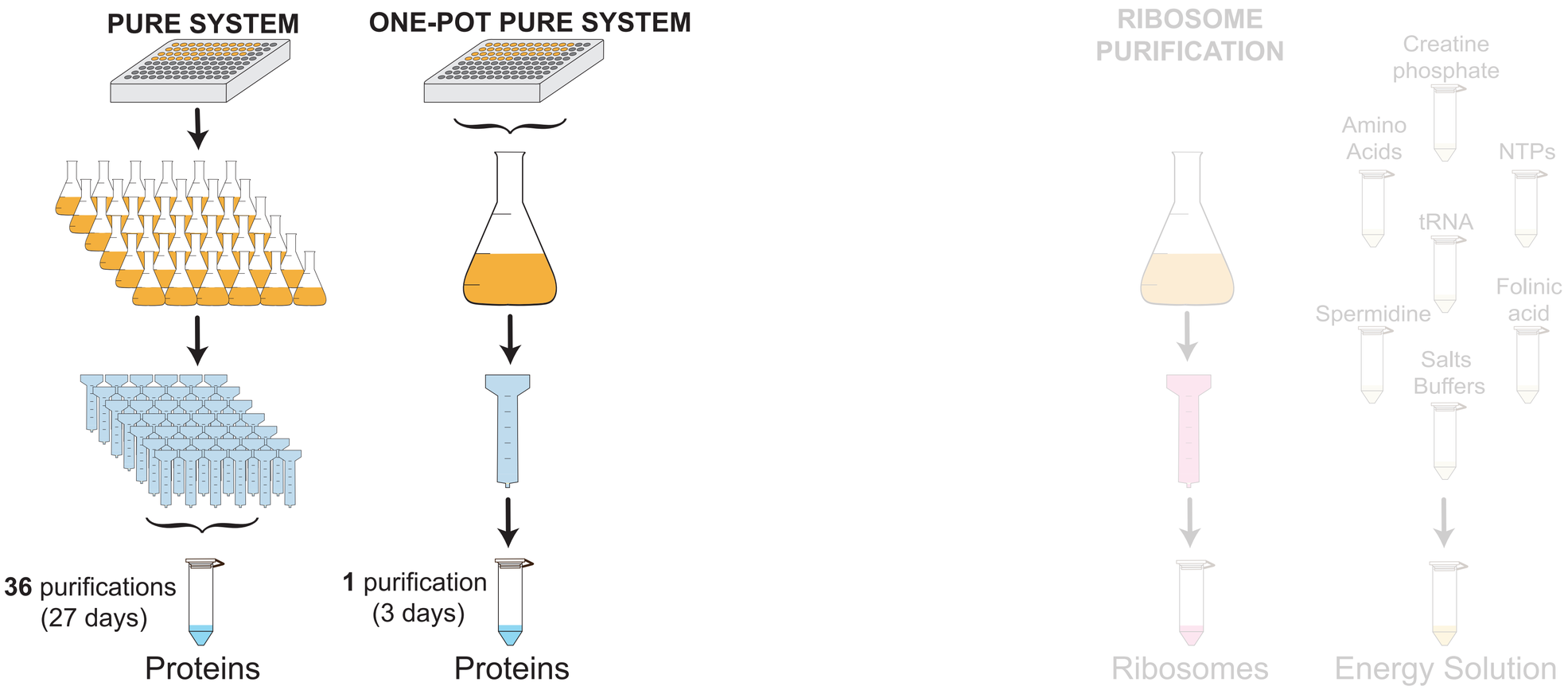
- Protocols
  - https://2019.igem.org/Team:EPFL/OnePot_Pure

- Other resources
  - https://www.addgene.org/browse/article/28197403/
  - https://www.addgene.org/browse/article/28196835/



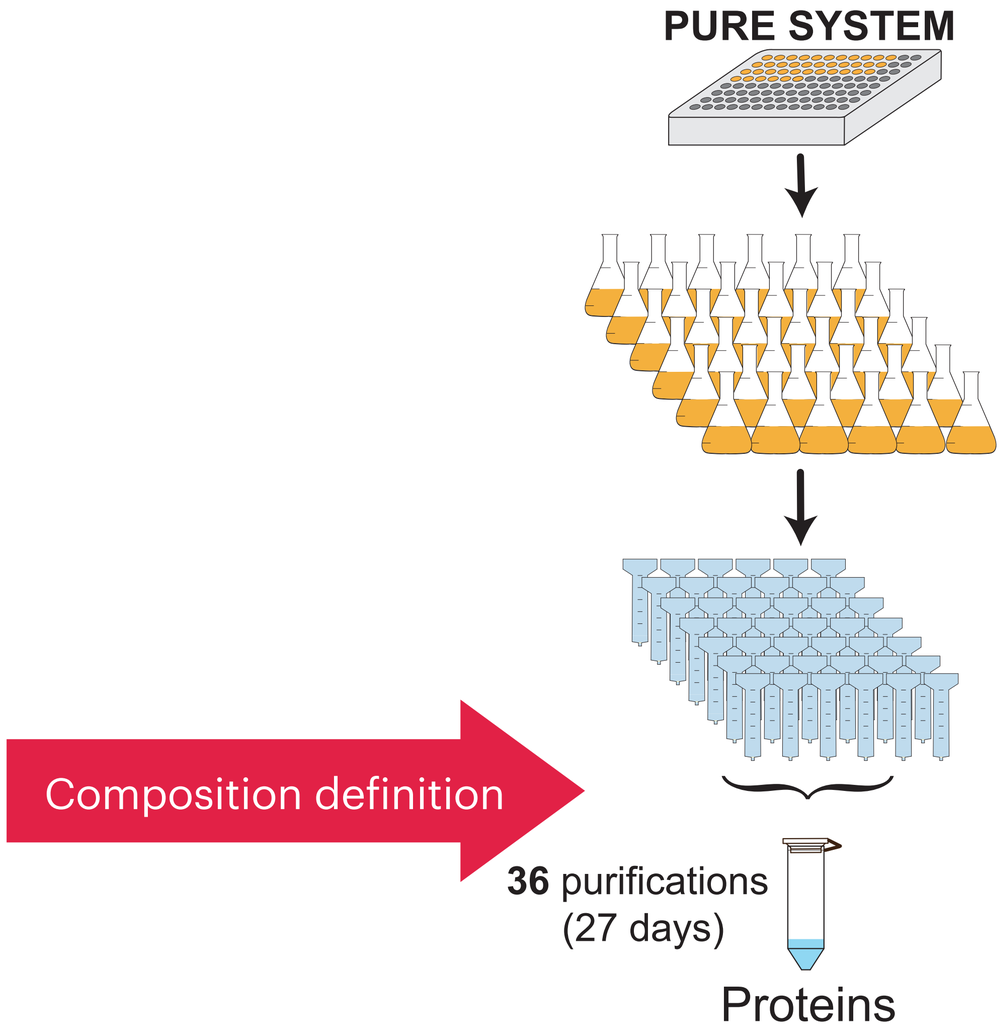
PURE / One-Pot PURE

Preparation



PURE / One-Pot PURE

Preparation

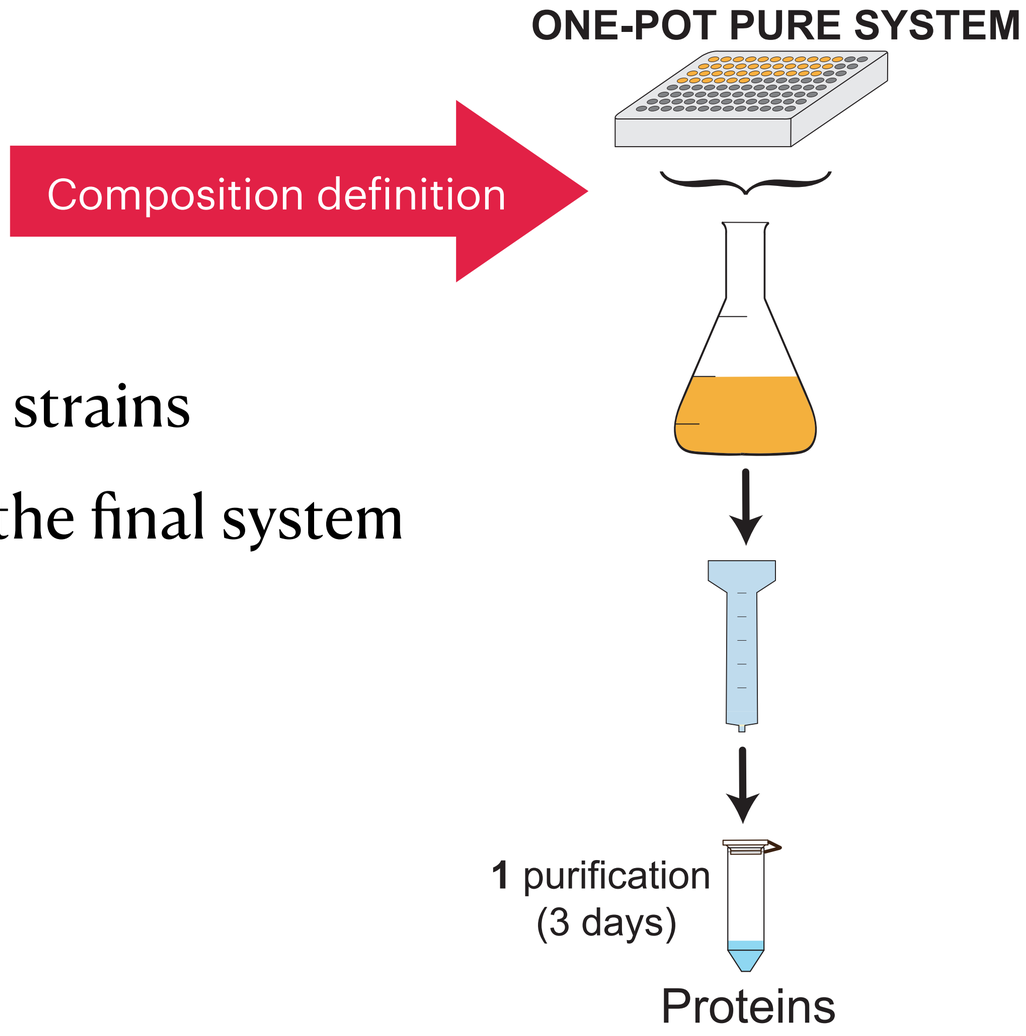


PURE / One-Pot PURE

Preparation

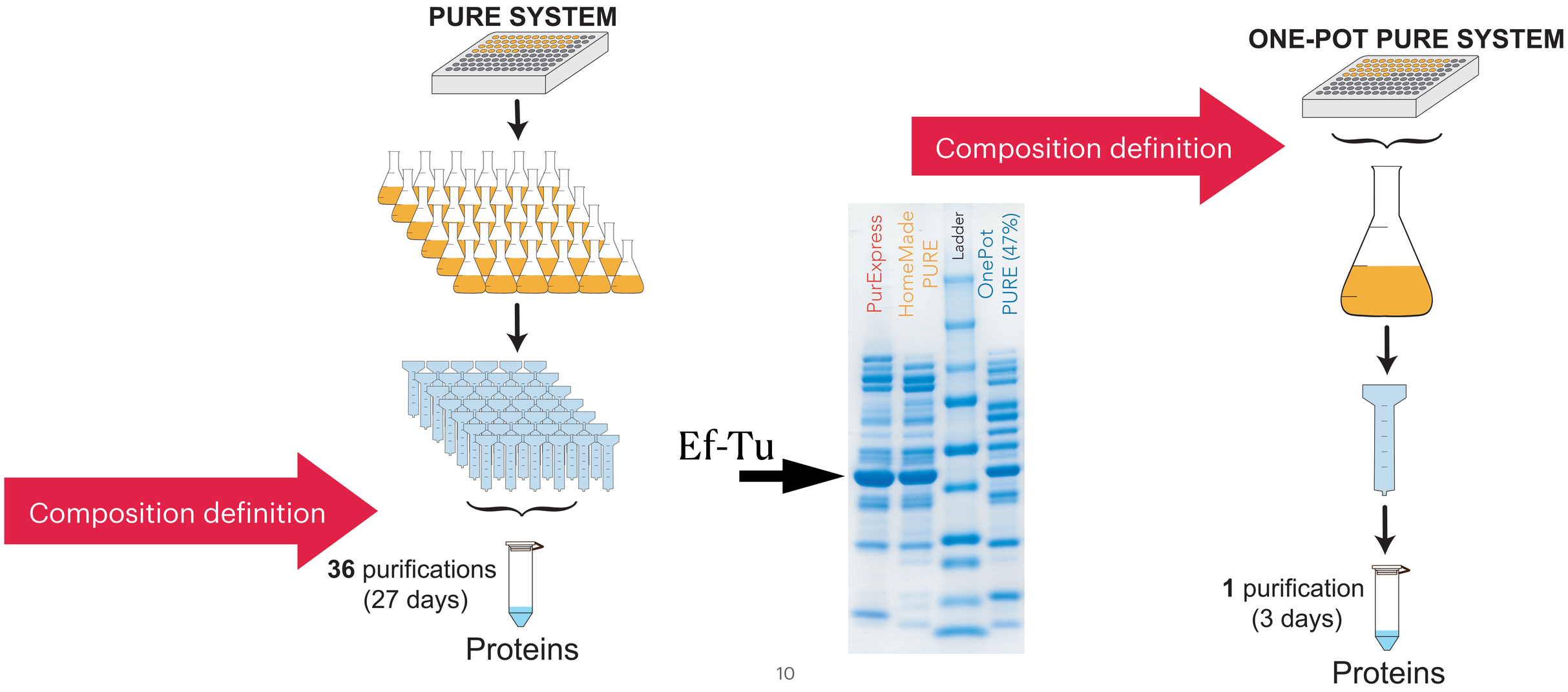
Ratio of the expression strains

~ amount of protein/enzyme in the final system



PURE / One-Pot PURE

Preparation



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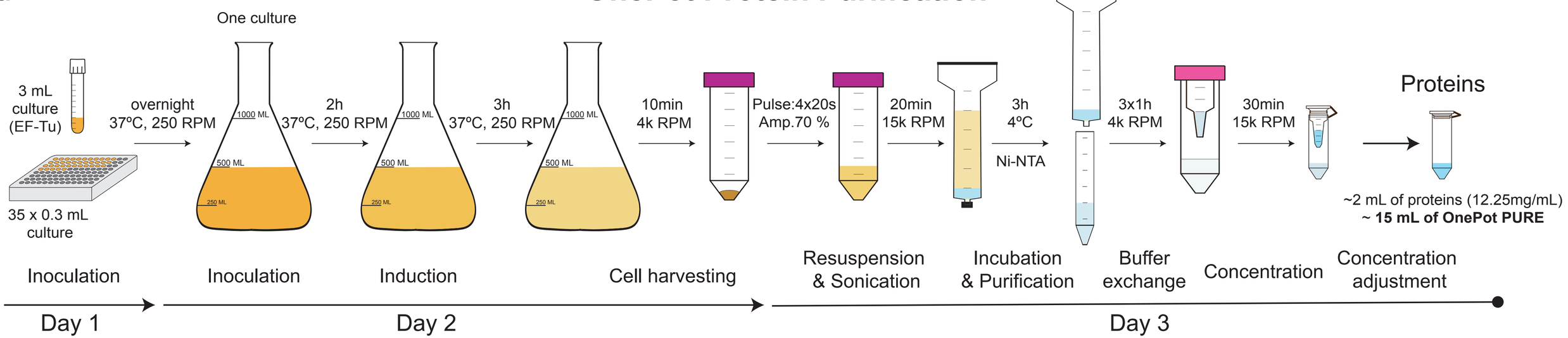
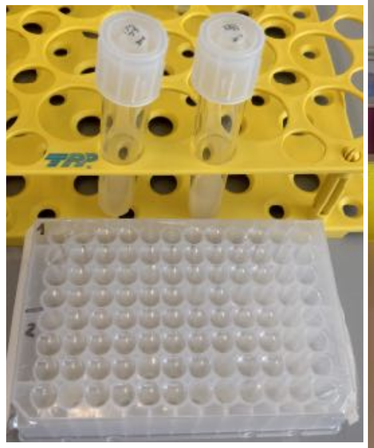
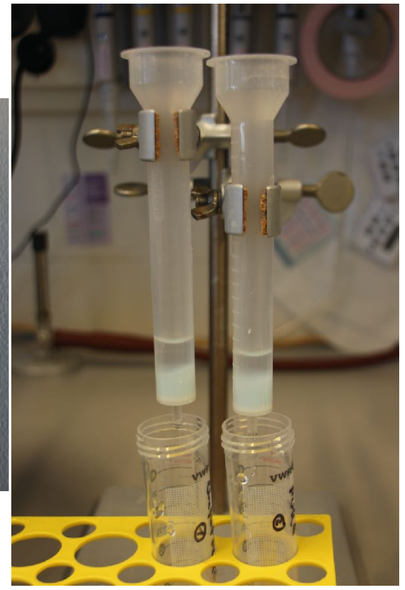
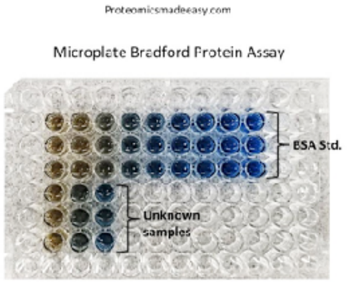
OnePot Protein Purification









On ICE!

buffer exchange or dialysis

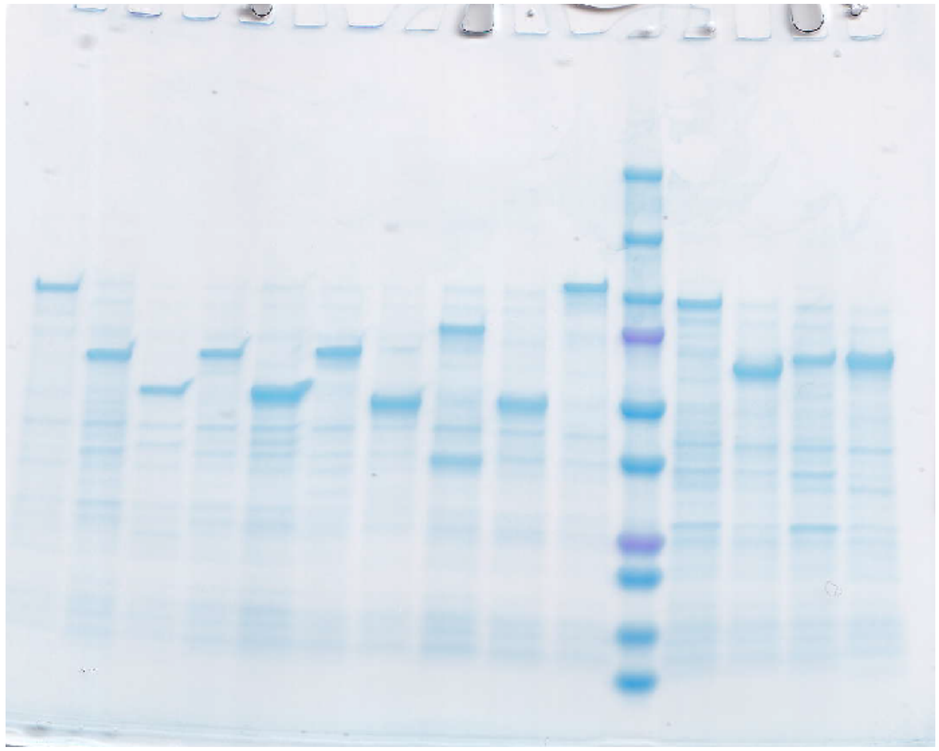
Strain preparation

- Correct strain
  - (https://openwetware.org/wiki/E._coli_genotypes)
  - JM109 + M15 - pQE vectors
  - DH5alfa or JM109 + BL21(DE3) - pET vectors

- Expression test

- Good growth
  - Oxygen
    - Breath-easy membrane
    - Hight rotation
    - Small volume or baffled flask

Expression test



1) Induce small cultures (200 -3000 μL)
2) mix 10 μL of culture with laemmli buffer
3) heat up, spin, add to gel

Preparation of PURE

Commercial (PURExpress, PureFlex)

✓ easy to use - protocols, additional supplements

✓ prepared - no additional work required

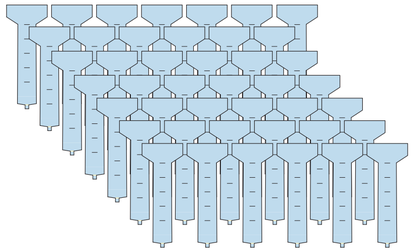
- expensive
- not modulable

Preparation: single components

✓ less expensive

✓ modulable

- labor intensive



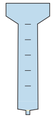
36 purifications
(27 days)

Preparation: single purification

✓ cheap

✓ easy to prepare

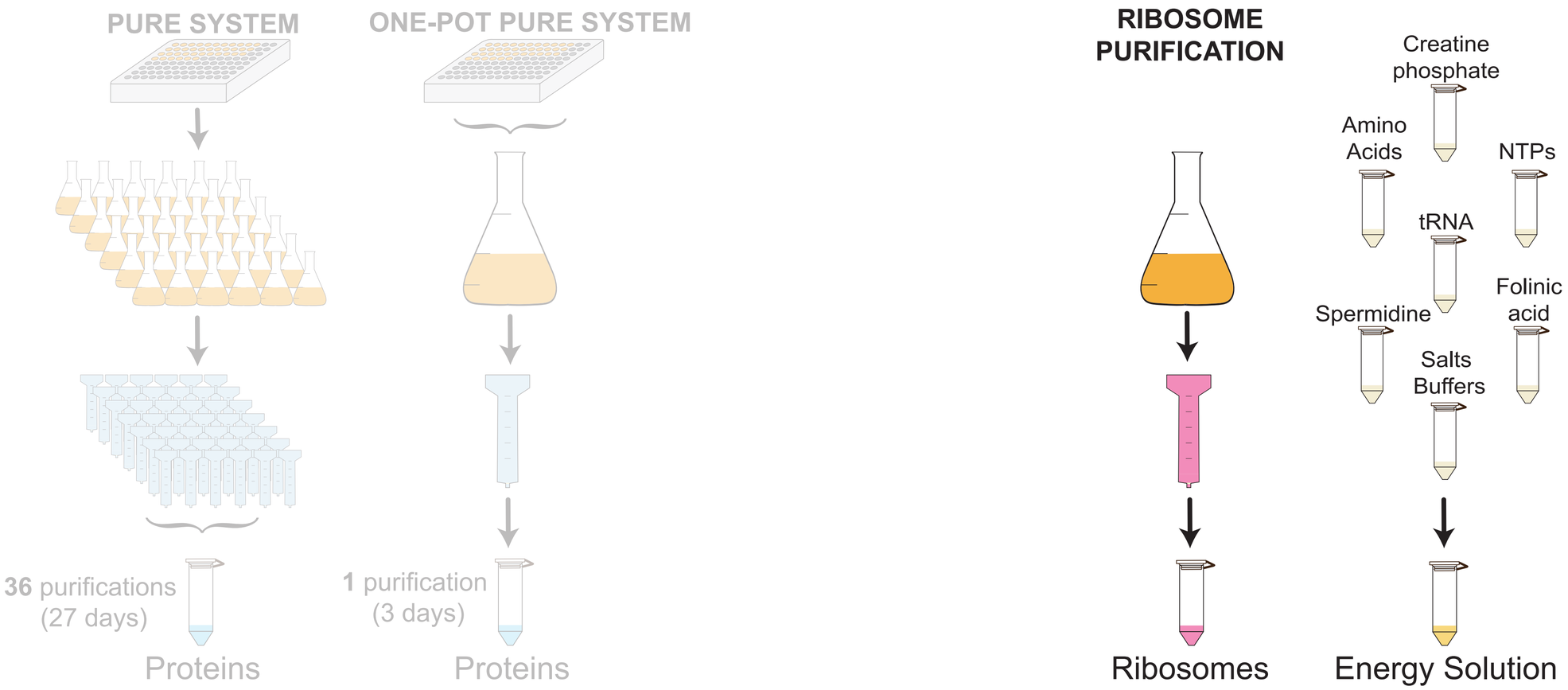
- less modulable



1 purification
(3 days)

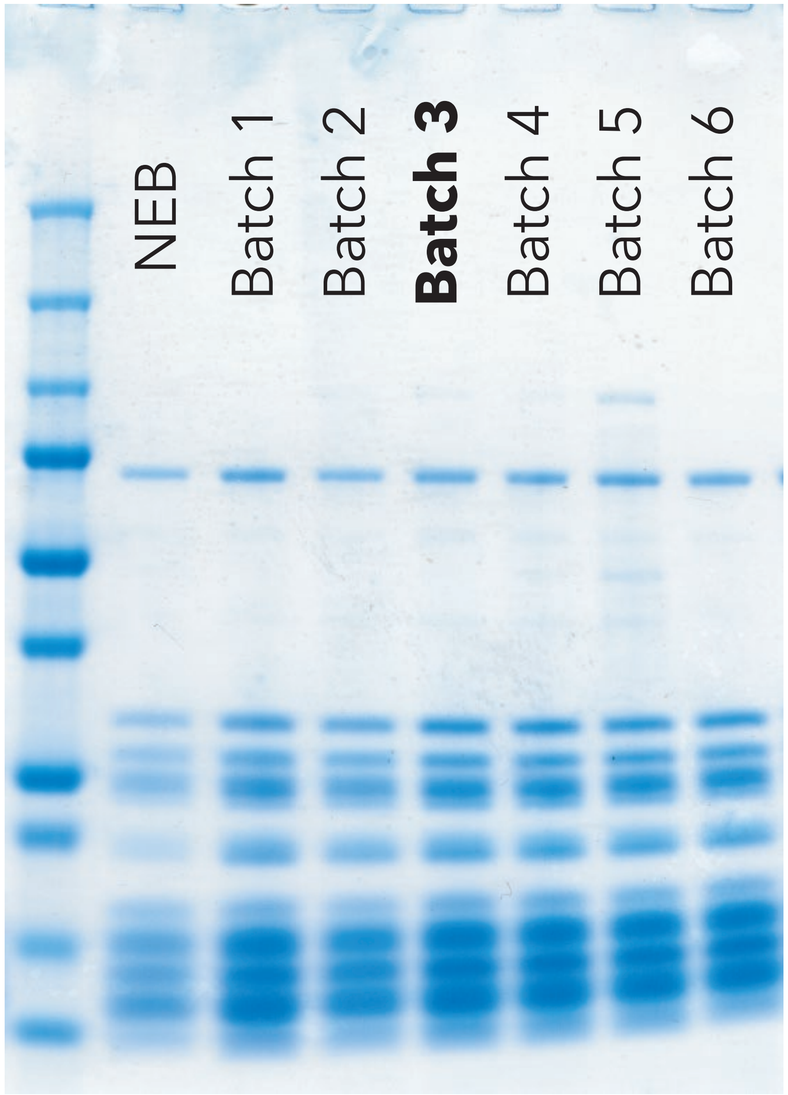
PURE / One-Pot PURE

Preparation



Ribosomes purification

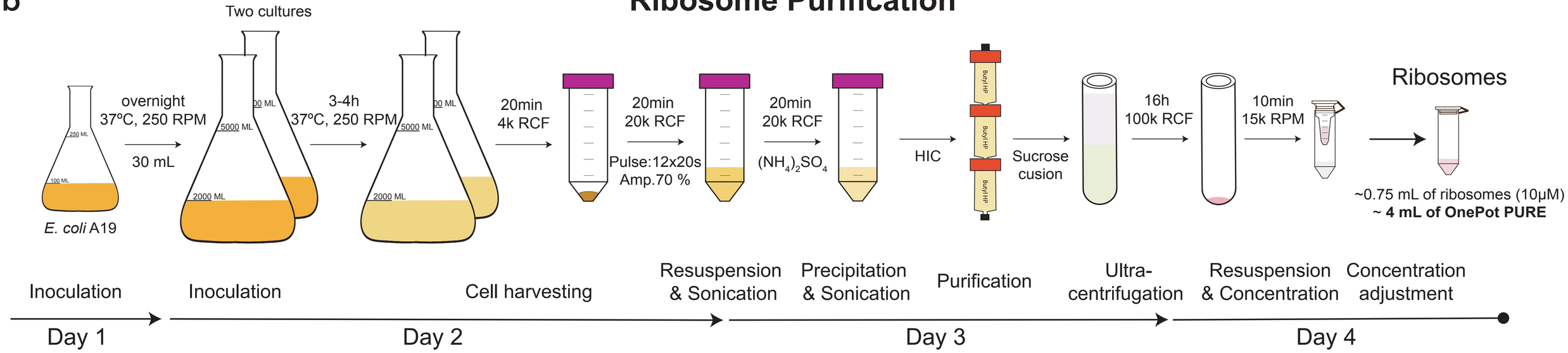
- No over-expression - just isolating already present ribosomes
  - A19 strain - RNase I deficient strain
- Ultracentrifugation sucrose cushion method
- His-tag ribosomes
  - Simple gravity flow purification
  - Not as productive when used in PURE
  - Multiplexed in Vivo His-Tagging of Enzyme Pathways for in Vitro Single-Pot Multienzyme Catalysis. Wang, 2012



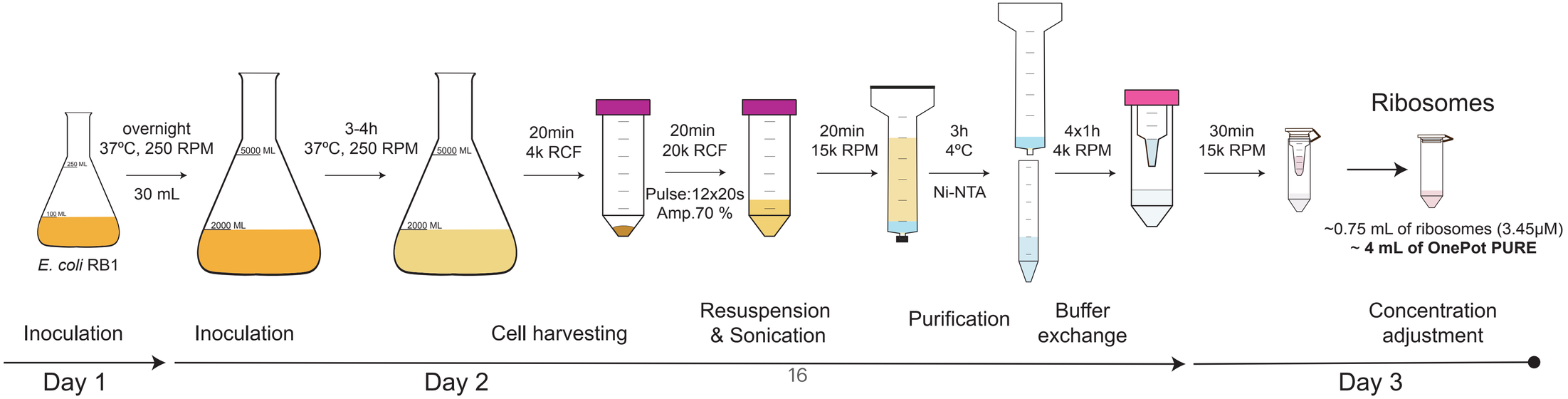
Purified Ribosomes (6.25µg)

b

Ribosome Purification



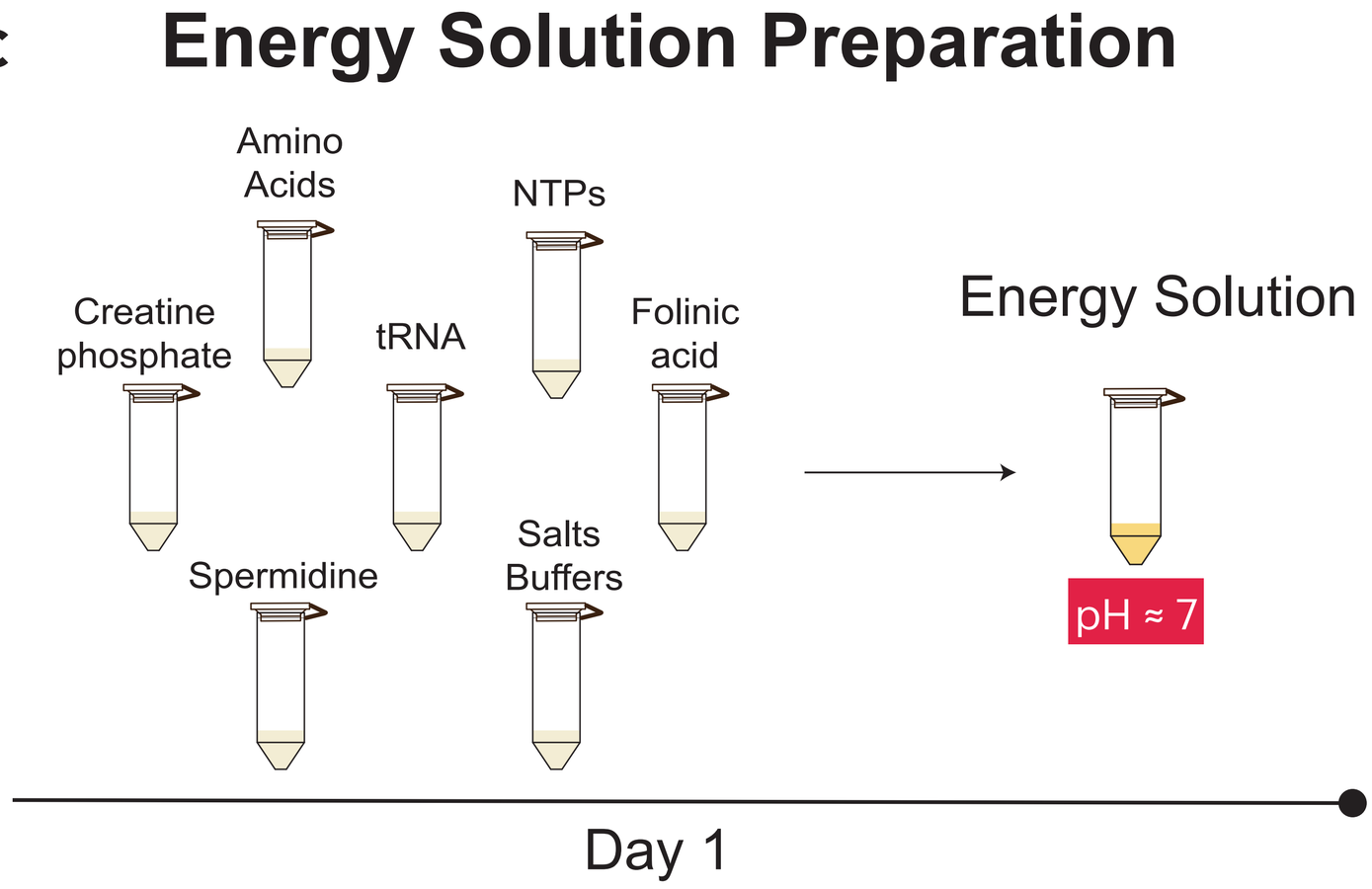
His-tag Ribosome Purification



PURE / One-Pot PURE

Preparation

c



Amino acid
- only in H_2O, no KOH

NTPs - NaOH buffered

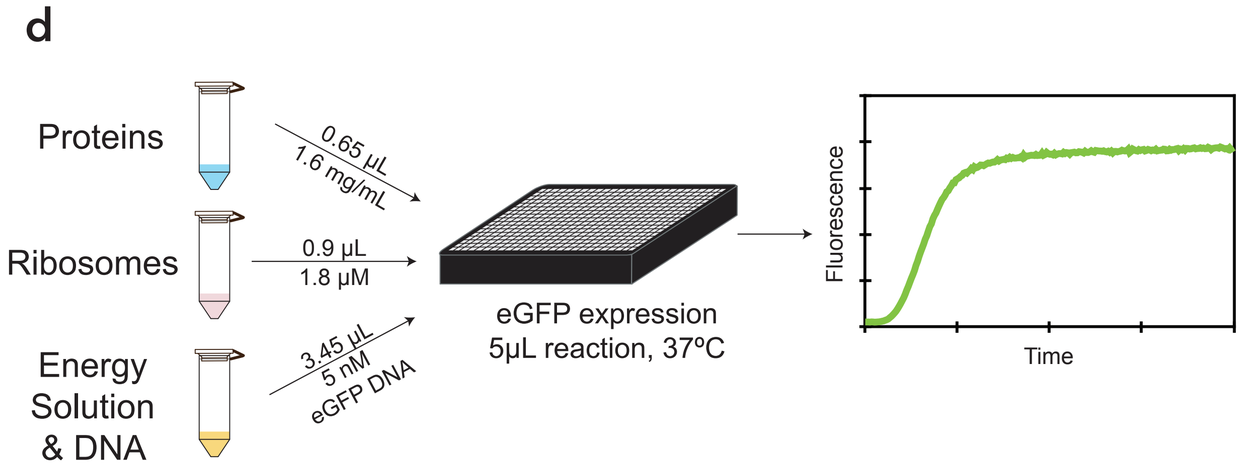
tRNA - add Nuclease-Free Water directly to the bottle

Buffers - use volumetric flasks

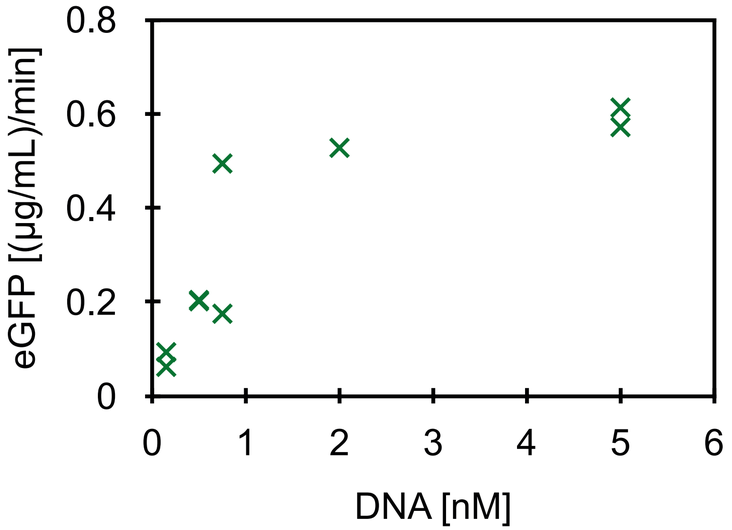
PURE / One-Pot PURE

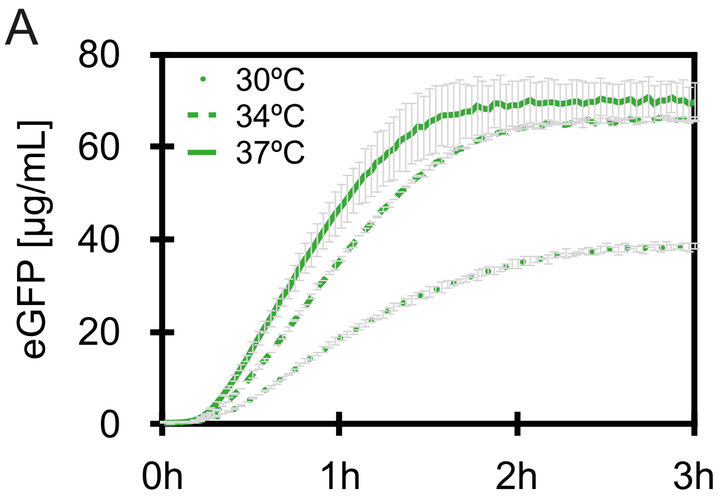
Testing

Platereader - fluorescence readout



Linear template - extension PCR

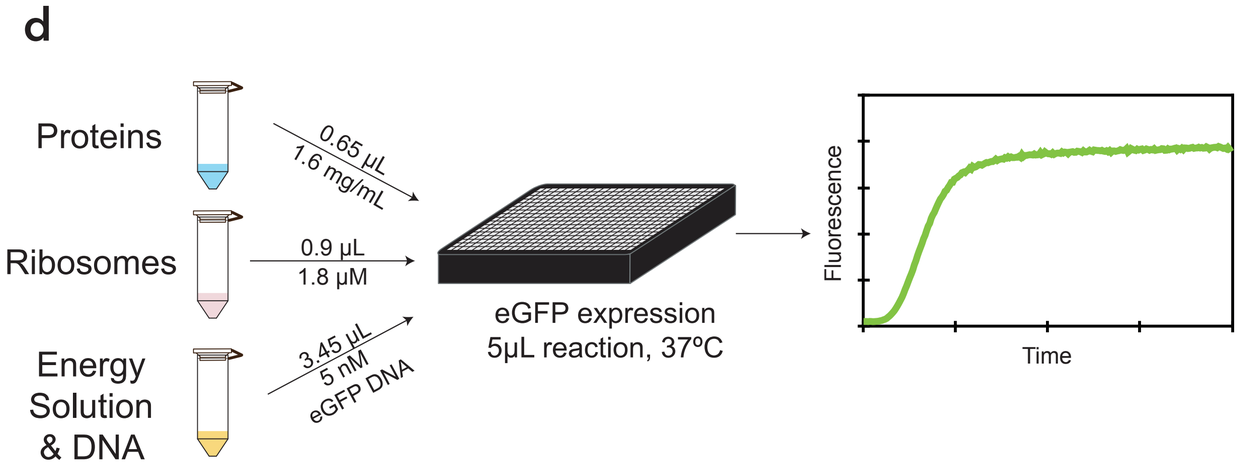




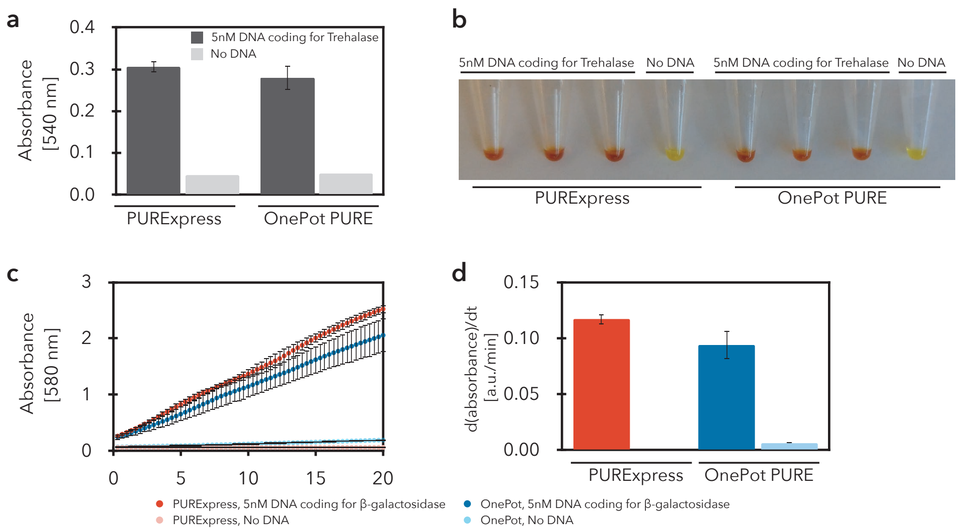
PURE / One-Pot PURE

Testing

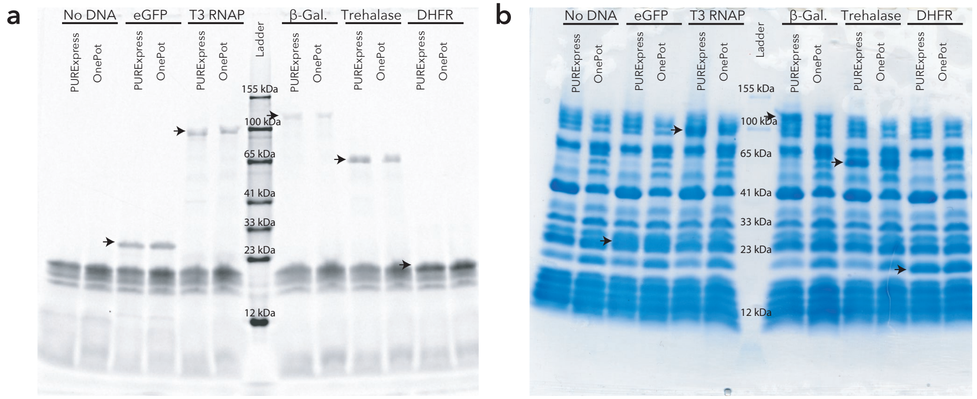
Platereader - fluorescence readout



Colorimetric detection



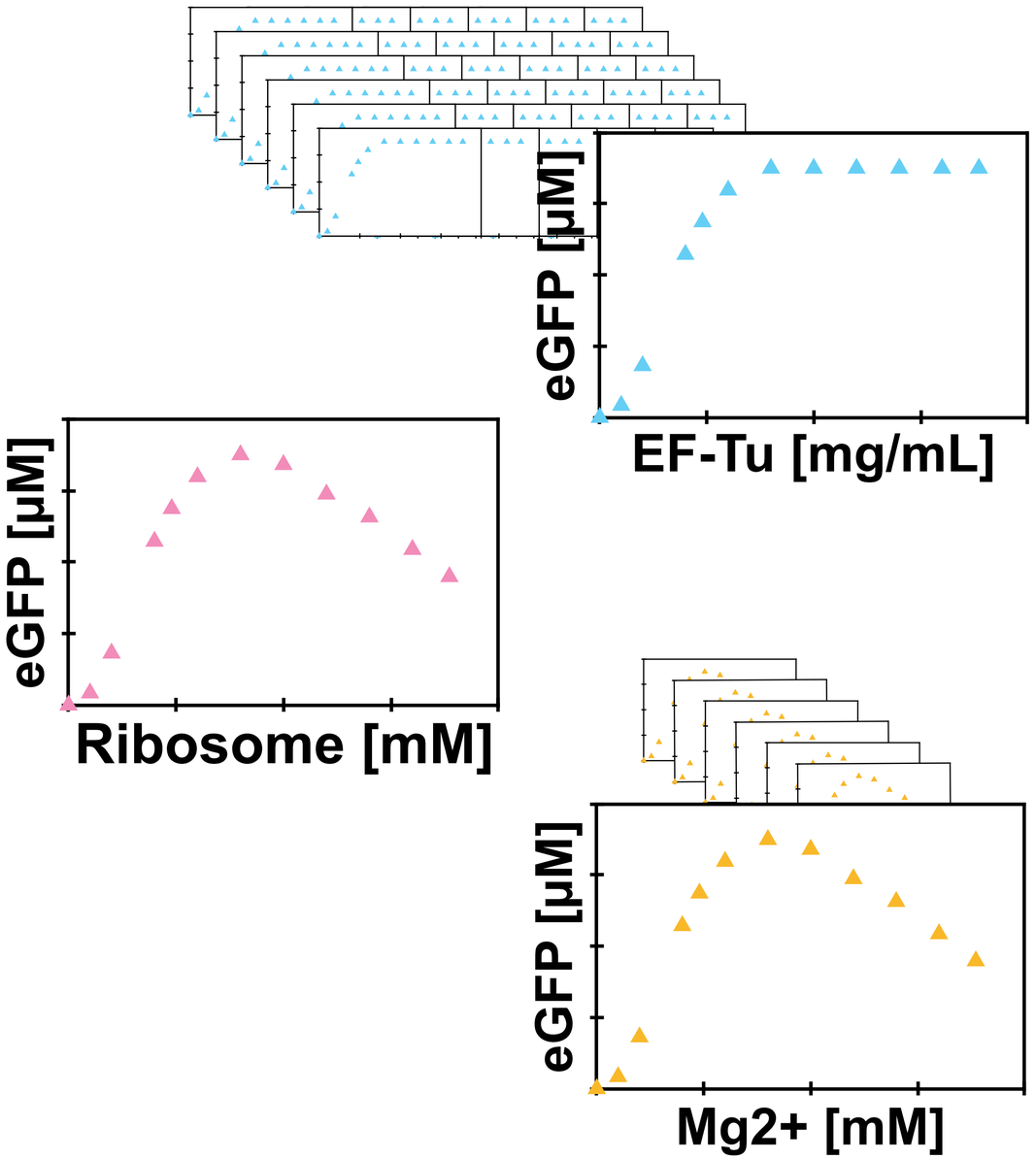
Gels



FluoroTect Green$_{Lys}$ tRNA

Important parameters

- EF-Tu
- Ribosomes
- Salts
  - Mg^{2+} (important for ribosome function)
    - Binds to NTP, creatine phosphate
- Redox reagent - FRESH!!
  - BME, DTT (do not use for HIS-purification), **TCEP**
  - Add to buffers just before use



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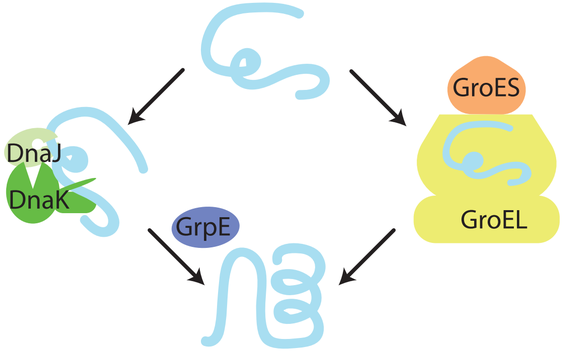
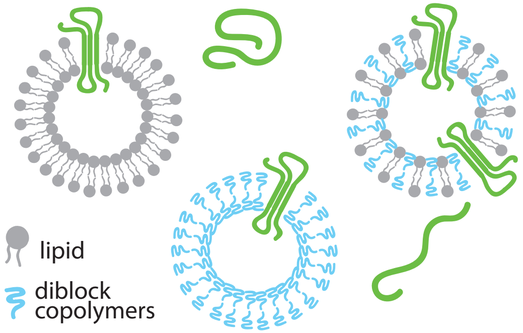
Post-translation

Protein folding



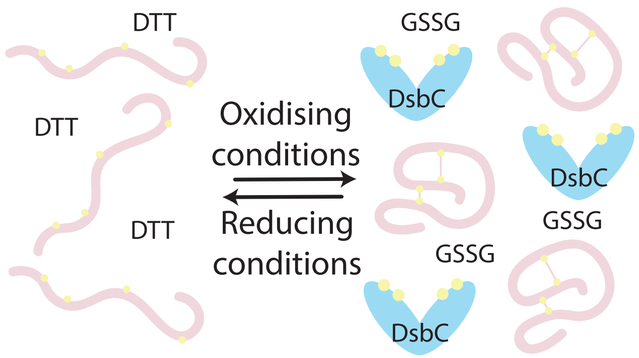
- Adding chaperones can help proteins to fold more efficiently

Membrane proteins



- Membrane proteins need vesicles to fold and function properly (most lysates contain vesicles)

Disulfide bonds



- Some protein need disulphide bonds to fold and function properly
- e.g. mammalian proteins - antibodies

Thank you for your attention.

Good luck to all of you.