LYTAG TWO-PHASE PURIFICATION SYSTEM

A scalable aqueous two-phase system to purify recombinant proteins by LYTAG affinity

Contents

1. About LYTAG Two-Phase Purification System.

LYTAG Two-Phase is a protein purification system based on the use of two aqueous components. The method relies on the affinity of the protein tag LYTAG for one of the two-phase components, allowing recombinant protein separation and purification from cellular extracts or culture media [1]. In the procedure, the LYTAG-fused protein is retained in one of the aqueous phases while most of the undesired proteins can be removed by simply discarding the opposite phase. After replenishing the system with fresh phase, the protein of interest can be easily recovered in it, with high purity, by reversing its localization with the addition of choline, the specific LYTAG ligand.

This system is particularly well suited for industries and laboratories specialized in protein separation and purification, as it is simple, cost efficient, time saving and highly versatile for scaling up protein purification processes, representing a convenient alternative to solid resins.

Main advantages of LYTAG Two-Phase Purification System:

1. Quick method. Separation can be completed within a few minutes, minimizing the effects of proteases. It is especially convenient when large volumes (>10 ml) of cellular extracts or culture media need to be processed (usually requiring prolonged flow times when using chromatography columns).

2. Easy and inexpensive protein purification process.

3. No special equipment requirements.

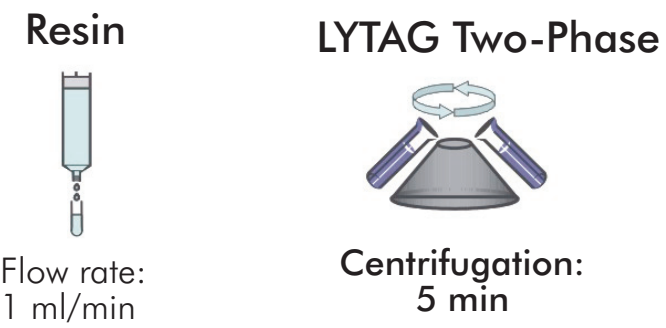
4. It offers, like every aqueous two-phase system, mild conditions in the separation of labile proteins. Purification can be easily and safely performed at low temperatures, requiring only a refrigerated centrifuge and an ice water bath.

5. Scalable method.

6. High purification efficiency (>95% purity), comparable to the use of solid matrices.

7. Optimum performance in downstream fermentation processes.

8. Good alternative to conventional solid resins.



Cell extract volumes	Resin	LYTAG Two-Phase
10 ml	30 - 60 min	15 - 30 min
50 ml	1,5 - 2 h	15 - 30 min
100 ml	3 - 4 h x4	15 - 30 min
200 ml	6 - 7 h x7	15 - 30 min

Figure 1. The LYTAG Two-Phase Purification System introduces a significant reduction in processing times, as compared to column chromatography, while avoiding flow-related problems associated with high viscosity and clogging.

The LYTAG-Two Phase Purification System Kit is available in two different sizes: for 20 ml cellular extract (Cat. No. KT-4697) and for 80 ml cellular extract (Cat. No. KT-4739). The number of purifications that can be performed with every kit size can be adjusted. Thus, several combinations are possible with the smaller kit (Cat. No. KT-4697), for example: 1 purification from 20 ml cellular extract, 2 purifications from 10 ml cellular extract, 4 purifications from 5 ml cellular extract , etc.

The components included are the followings:

Reagents provided	Size				Storage [c]
	for 20 ml cellular extract		for 80 ml cellular extract		
	QUANTITY	CAT. Nº	Quantity	CAT. Nº	
Top Solution	20 ml	RS-4751	80 ml	RS-4740	RT [d]
Lower Solution	10 ml	RS-4752	40 ml	RS-4741	RT
LYTAG Two-Phase Wash Buffer(2x)[a]	65 ml	RS-4753	240 ml	RS-4754	RT
3 M Choline Chloride	10 ml	RS-4755	25 ml	RS-4756	4°C
1M Salicylate (Inducer)	25 ml	RS-3247	25 ml	RS-3247	4°C
pALEX2a pALEX2b pALEX2c	8 µg (each)	EV-4658	8 ug (each)	EV-4658	-20°C
pALEX2-Ca-GFP [b] (expression and purification control plasmid)	8 µg	EV-4757	8 µg	EV-4757	-20°C
E. coli REG12	Stab	BS-3458	Stab	BS-3458	4°C

(a) The provided LYTAG Two-Phase Wash Buffer is 2-time concentrated. Dilution with distilled water prior to use is required (1 volume LYTAG Two-Phase Wash (2x) : 1 volume distilled water).

(b) The recombinant fluorescent protein GFP-LYTAG is expressed under salicylate induction of the CASCADE™ REG12 *E.coli* strain transformed with pALEX2-Ca-GFP plasmid. A control LYTAG Two-Phase purification assay can be performed with this transformant strain as recombinant protein presence can be easily followed by yellow-green color.

(c) LYTAG Two-Phase Purification System kit is shipped at RT. Upon arrival, store the components at the temperatures recommended in the table above. It is strongly recommended to keep and work with the solutions under sterile conditions, especially the Top Solution and LYTAG Two-Phase Wash Buffer (2x).

(d) RT = Room temperature.

Equipment and reagents required but not provided:

- Lysis buffer (20 mM potassium phosphate buffer pH 8.0 is recommended).
- Centrifuge capable of 10,000 x g.
- Centrifuge tubes (transparent tubes are recommended).

2. About the purification tag (LYTAG) and the expression system.

LYTAG is a polypeptide sequence convenient for the efficient recombinant expression and purification of proteins in *E. coli*. This system employs a reduced (136 aa, 15.21 KDa) version of the choline binding domain of the *Streptococcus pneumoniae* N-acetylmuramoyl-L-alanine amidase LytA (C-LytA) that has strong affinity for choline or choline analogues (tertiary or quaternary amines) [2, 3]. The tertiary structure of C-LytA has been resolved [4].

The LYTAG system yields high expression levels when induced, but maintains reduced basal levels prior to induction. It integrates the CASCADE™* technology, in which the expression of the fusion protein is controlled by linked regulatory circuits to amplify gene expression (Figure 2) [5].

CASCADE™ employs two salicylate-responsive transcriptional activator proteins, NahR and XylS. In the presence of salicylate, the NahR protein induces expression of XylS from the P_{sal} promoter. Salicylate also activates XylS, which induces high-level expression of the gene of interest from its cognate *Pm* promoter. The synergistic effect of using two transcriptional regulators in a sequential cascade amplifies expression levels nearly 20-fold compared to expression from either promoter individually.

The regulatory module of the cascade system (*nahR/Psal::XylS*) is located in the chromosome of the *E. coli* REG-12 expression strain and the expression module (*Pm: LYTAG*) is contained in the pALEX2 vectors.

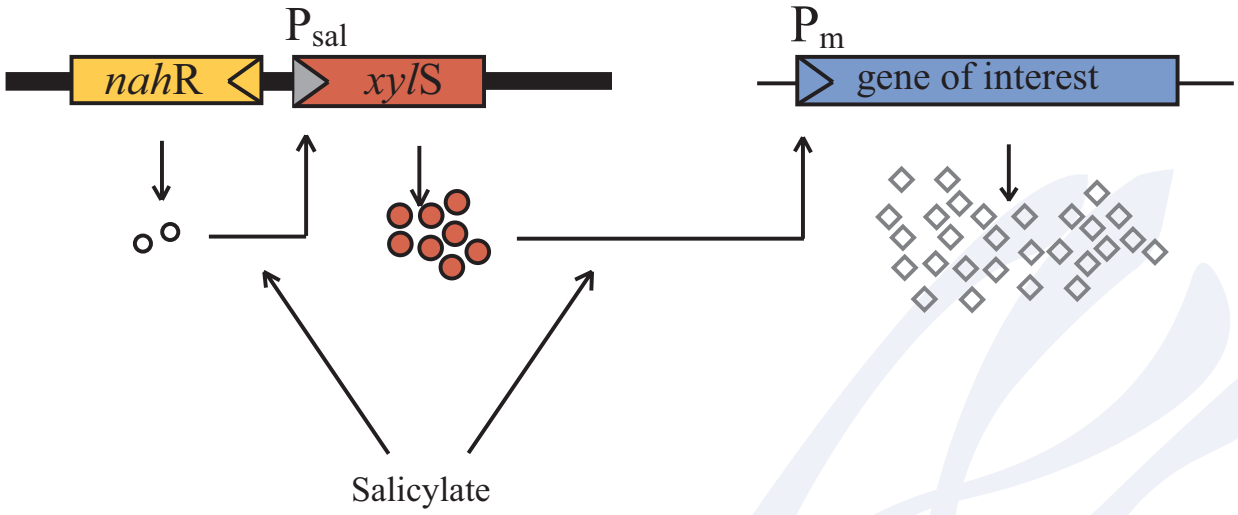


Figure 2. Cascade™ Expression system

* CASCADE™ is a trademark of Active Motif, Inc., Carlsbad. The CASCADE™ expression system is patent pending and licensed by Active Motif, Inc. Commercial license available. Please contact us if you want more information about license agreement.

2.1. Strain and vectors

2.1.1. *E.coli* REG-12

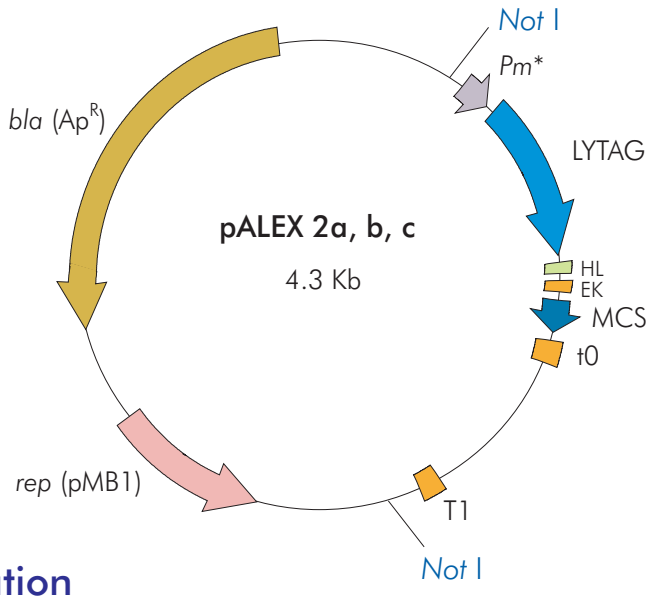
REG-12 is a rifampicin-resistant (Rif^R) *E.coli* K12 mutant that contains the regulatory element *nahR/Psal::xylS* integrated in the chromosome [6]. This strain is a fast growing *E. coli* that can host the CASCADE expression vectors [5]. REG-12 can reach high cell density in rich media and can also grow in minimal media with ammonia and glucose as nitrogen and carbon sources, respectively.

Genotype

F^+ mini-Tn5 (kan^R nahR/Psal::xylS) Rif^R

2.1.2. pALEX vectors

pALEX2 map



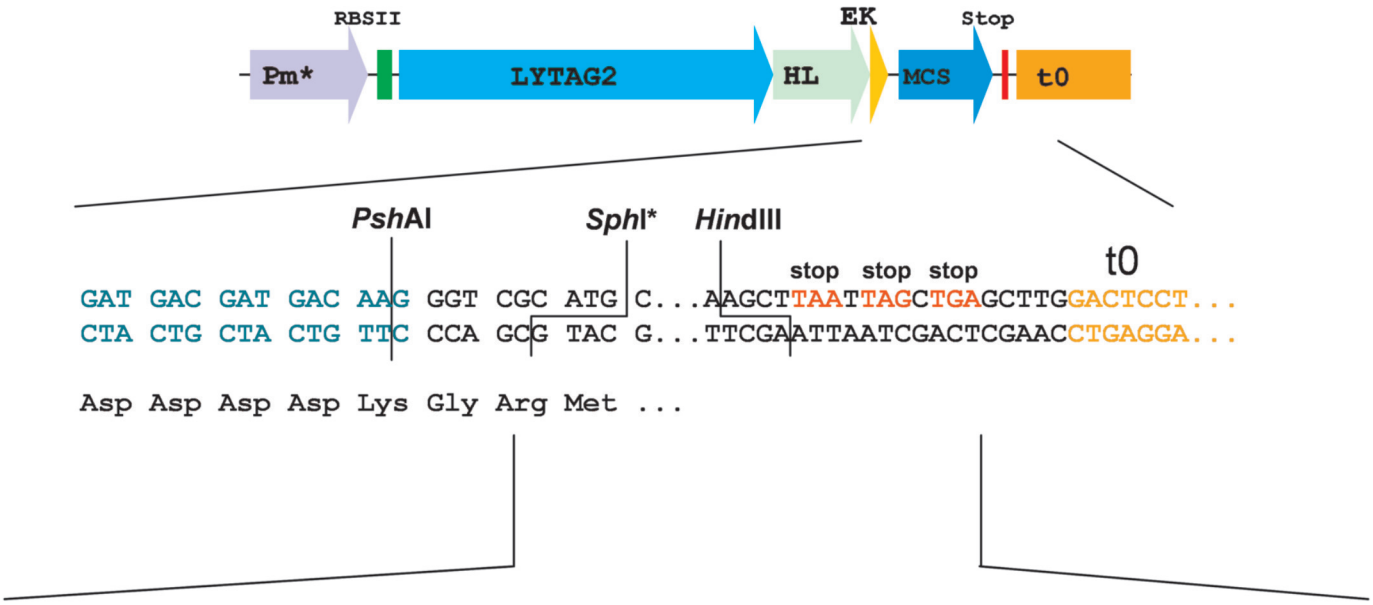
General information

The new pALEX2 vector series is designed to be used with the CASCADE™ host strains for the expression, purification and immobilization of proteins fused in their N-termini to a shortened and improved version of the choline binding protein, LYTAG, using the LYTRAP chromatographic resin and its related reagents. The new LYTAG (136 aa, 15.21 KDa) incorporates an alpha helix polypeptidic sequence which acts as a spacing element between LYTAG and the fused protein, reducing the potential steric interference between both regions and facilitating elimination of the tag by enterokinase digestion.

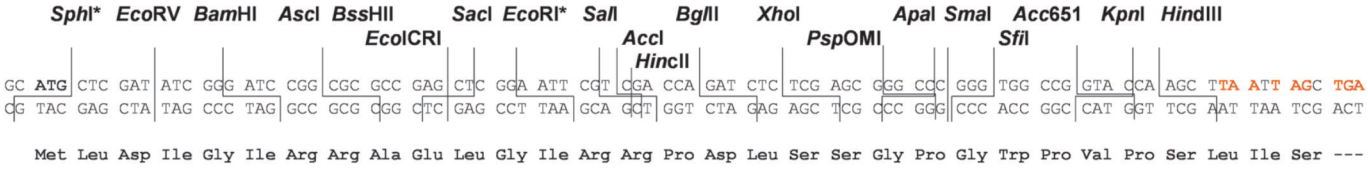
Among other features, pALEX2a, b and c incorporate a mutant *Pm* promoter with even lower basal activity and have an improved multiple cloning site (MCS), for easy and flexible cloning in-frame with the LYTAG coding sequence, as well as stop codons in all three forward reading frames, followed by strong transcriptional terminator sequences (t0 and T1).

Another interesting feature is the presence of a unique restriction site for the blunt cutter *Psh*AI overlapping with the sequence encoding the enterokinase recognition site, allowing its direct fusion with the protein of interest and recovery of a product without undesired N-terminal amino acid residues, after enterokinase cleavage. In addition, the expression cassette in pALEX2a, b and c is flanked by "rare cutter" *Not*I sites, for convenient subcloning into mini Tn5 delivery vectors and construction of genetically stable expression strains.

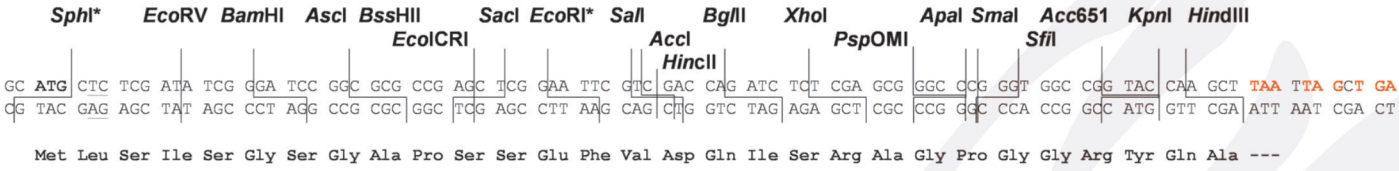
Sequence and restriction analysis



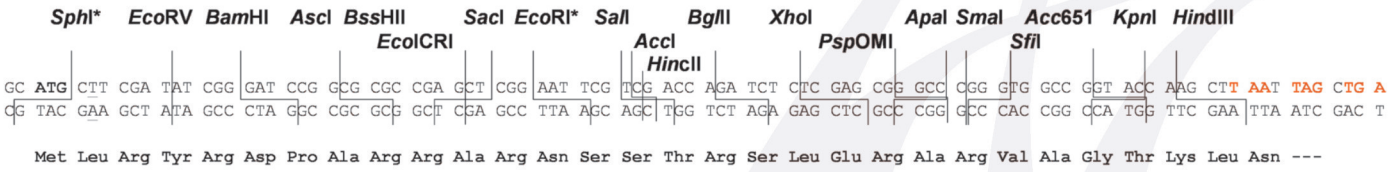
pALEX2a



pALEX2b



pALEX2c



RESTRICTION ENZYMES THAT DO NOT CUT pALEX2

AarI	*AbsI*	*AfeI*	*AflII*	*AgeI*	*AjuI*	*AleI*	*AlfI*	*AloI*
AsiSI	*AvrII*	*BbvCI*	*BclI*	*BmgBI*	*BplI*	*BsaAI*	*BsabI*	*BsgI*
BsiWI	*BsmFI*	*BspMI*	*BsrGI*	*BstBI*	*BstEII*	*BstXI*	*BstZ17I*	
ClaI	*CspCI*	*DraIII*	*EcoNI*	*FalI*	*FseI*	*FspAI*	*HpaI*	*MfeI*
MluI	*MreI*	*NaeI*	*NgoMIV*	*NruI*	*NsiI*	*PacI*	*PmeI*	*PmlI*
PpuMI	*PsiI*	*PspXI*	*PsrI*	*PstI*	*RsrII*	*SacII*	*SanDI*	*SbfI*
SexAI	*SgrAI*							

RESTRICTION ENZYMES THAT CUT ONCE pALEX2 vectors

AatII	*Acc65I*	*AccI*	*AcuI*	*AflIII*	*AhdI*	*AlwNI*	*ApaI*	*AscI*
BaeI	*BamHI*	*BbsI*	*BglII*	*BlpI*	*BmtI*	*Bpu10I*	*BsaI*	*BsaXI*
BseRI	*BsmBI*	*BspEI*	*BssHII*	*BstAPI*	*Bsu36I*	*BtgZI*	*EcoICRI*	
Eco0109I		*EcoRV*	*HincII*	*HindIII*	*KpnI*	*MscI*	*NdeI*	*NheI*
PasI	*PciI*	*PflMI*	*PfoI*	*PshAI*	*PspOMI*	*SacI*	*SalI*	*SapI*
SfiI	*SmaI*	*StuI*	*XbaI*	*XcmI*	*XhoI*	*XmaI*	*XmnI*	*ZraI*

pALEX2-Ca-GFP plasmid

The pALEX2-Ca-GFP plasmid can be used as positive control in induction and purification assays.

The recombinant fluorescent protein GFP-LYTAG is expressed under salicylate induction of the CASCADE™ REG12 *E.coli* strain transformed with pALEX2-Ca-GFP plasmid. A control LYTAG Two-Phase purification assay can be performed with this transformant strain as recombinant protein presence can be easily followed by the yellow color of the GFP.

3. Procedures

3.1. Cloning into pALEX2 vectors and host strain transformation (*E. coli* REG-12)

The cloning strategy must take into consideration that the DNA encoding the protein of interest must be cloned in frame with the LYTAG coding sequence (see LYTAG and MCS sequence). Determine which restriction sites will be used for cloning and then choose the pALEX2 vector that will preserve the reading frame at the 5' end.

Transformation of *E. coli* REG-12 strain may be performed using standard methods. Biomedal recommends TSS solution (Cat. No. RS-3215/16), a fast and simple system to prepare competent cells (for more information, visit www.biomedal.com).

3.2. Protein Expression

Optimal expression conditions should be determined for each particular recombinant protein with small scale cultures before attempting large scale expression procedures. Expression can be optimized by varying inducer concentration (0.02-2.0 mM salicylate), temperature (16-37°C), time (1h-overnight), and OD_{600} of induction (0.2-1.0). The following can be a start point expression protocol:

1. Inoculate a pre-culture with a single, freshly transformed colony. Add the appropriate antibiotics (100 µg/ml ampicillin and, optionally, 25 µg/ml kanamycin) to the pre-culture and incubate overnight at 37°C with shaking (225 rpm).

2. Dilute pre-culture 1:100 in fresh ampicillin-containing medium and continue incubation in the same conditions until OD_{600} reaches 0.7-1.0.

3. Add the expression inducer (salicylate) to a final concentration of 2 mM. Incubate 5 h at 30°C with shaking (225 rpm).

3.3. Cellular Extract

1. Harvest cells. Pellet the culture overexpressing the recombinant protein by centrifugation at 4,000 x g for 15 min at 4°C. Remove the supernatant and store the pellet at -80°C until needed.
2. Cell disruption. Resuspend the pellet in lysis buffer (recommended 20 mM potassium phosphate buffer pH8.0). Standard *culture volume : lysis buffer* ratios are among *25:1 – 10:1*. Keep the sample on ice and disrupt the cells by sonication or using a French press.
3. Centrifuge at 9,000 x g for 20 min to pellet cell debris.
4. Remove the supernatant (crude lysate) to a fresh container and keep on ice.

3.4. Protein Purification

3.4.1. Standard Protocol

1. Use a transparent centrifuge tube to mix 1 volume of crude lysate with 1 volume of Top Solution and 0.5 volumes of Lower Solution. Mix thoroughly by inverting the tube to obtain a homogeneous suspension.
2. Centrifuge at 10,000 x g for 5 min, 4 °C.
3. Using a pipette, transfer the upper phase to a clean centrifuge tube. Discard the lower phase.
4. Wash[a]. Prepare 1x LYTAG Two-Phase Wash Solution by diluting the provided 2x stock in distilled water. Determine the volume of the recovered upper phase and add to it 1.5 volumes of 1x Wash Solution. Mix thoroughly by inverting the tube and centrifuge at 10,000 x g for 5 min, 4 °C.
5. Repeat Step 3.
6. Elution to lower phase. Prepare Elution Solution by diluting the provided 3M Choline Chloride solution in LYTAG Two-Phase Wash Buffer 2x (0.5 volumes LYTAG Two-Phase Wash (2x) : 0.1 volumes 3M Choline Chloride : 0.4 volumes distilled water). Determine the volume of the recovered upper phase and add to it 1.5 volumes of Elution Solution. Mix thoroughly by inverting the tube and centrifuge immediately at 10,000 x g for 5 min, 4°C. Transfer the upper phase to a different tube and check for the presence of the recombinant protein in the lower phase.

(a) Wash Steps 3 and 4 may be repeated if a higher degree of purity is required.

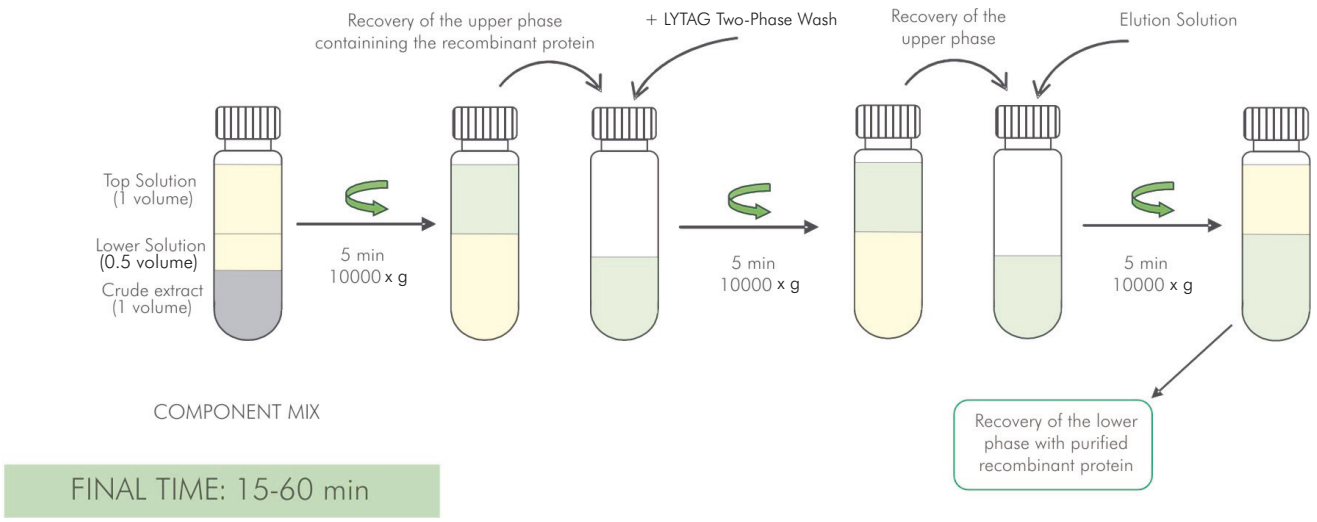


Figure 3. General procedure. Processing time is independent of initial lysate volume.

3.4.2. Adapted Protocol to low protein expression levels

In some cases, when a high overexpression level of the recombinant protein can not be achieved, it may be convenient to reduce the relative volume of the upper phase during the first step of the purification procedure, in order to increase the concentration of the protein in this phase and hence in the elution fraction at the end of the process. To this purpose, in Step 1 of the procedure, mix 1 volume of crude lysate with 0.5 volumes of distilled water, 0.2 volume of Top Solution and 0.8 volume of Lower Solution and centrifugue as in Step 2. After phase separation, the upper phase will represent about 10% of the total volume. For the remaining protocol, proceed to Step 3 and beyond as described in the Standard Protocol (3.4.1.)

3.5. Protein Analysis

Samples from every purification step can be analyzed by SDS-PAGE. However, migration of samples coming from upper and lower phases can be affected by the high concentration of the two-phase components. Specifically, proteins in the upper-phase samples will tend to migrate with an apparent molecular weight lower than expected. To avoid band distortion and facilitate proper migration, several alternative procedures are proposed:

1. Samples can be dialyzed prior to analysis by SDS-PAGE and Coomasie staining.
2. Samples can be diluted (50-fold minimum) prior to SDS-PAGE. In this case, it may be necessary to perform silver staining to increase detection sensitivity. By diluting the samples, the effect of the two-phase components in electrophoresis is minimized.
3. Alternatively, samples can be diluted only 5 times. In this case, take in account that upper-phase samples migrate showing an apparent molecular weight lower than expected, which can be compensated by adding Top Solution to the lower-phase samples and to the molecular weight marker. As an example of sample preparation with this strategy, mix 10 µl of lower-phase sample with 8 µl of Top Solution and 32 µl of 1.5x protein loading buffer.

The recombinant protein is eluted to the lower phase by choline addition with a high purity degree (Step 6). In some cases, dialysis of the eluted protein may be required due to the substantial salt concentration in lower phase.

3.6. General Notes

• LYTAG Two-Phase Purification System allows purifications from small volumes of cell lysate. However, in order to achieve maximum yields, it is advisable to begin with a 4-5 ml lysate volume, which will minimize volume (and protein) losses during phase transfer in the procedures.

• In order to optimize the phase recovering, transparent tubes with a small cut section surface are recommended. For example, use 15 ml conical tubes for purification from 4-6 ml lysates and 50 ml conical tubes for purification from 15-20 ml lysates.

• To separate the phases in Steps 2, 4 and 6, lower centrifugation speeds are suitable, being possible to work at 2,500 x g if special mild conditions are necessary. Furthermore, centrifugation can be performed at room temperature, instead of 4°C.

• In some cases, a solid precipitate may settle in the interphase after phase separation in Step 2. This precipitate contains mainly impurities and should be discarded together with the lower phase.

4. Troubleshooting

Problem	Possible cause	Solution
No expresssion or low expression levels	Gene is cloned into the wrong reading frame.	Review your cloning strategy to ensure that you choose the correct a, b or c vector.
	Culture temperature during induction is too high or too low.	Change induction temperature (16-37°C).
	Concentration of inducer is too low.	Increase the concentration of inducer (0.5-2.0 mM salicylate).
	Messenger RNA instability or problems with translation (presence of multiple rare codons in the gene of interest).	Overexpression of the corresponding tRNA can help.
	Insufficient cell disruption.	Check cell disruption conditions.
Improper protein folding	Culture temperature during induction is too high.	Use a lower temperature (20°C or less).
No fusion protein is detected by SDS-PAGE in any of the phase samples	Electrophoretic migration is distorted by the high two-phase component concentration.	Dilute the samples 50 times prior to electrophoresis and perform silver staining. Alternatively, dialyze or desalt samples with standard protocols prior to loading the gel.
	Expression level is too low and consequently, the purified protein is too diluted.	Perform the *Adapted Protocol* to concentrate the recombinant protein.
		Dilute the samples 5-fold to electrophoresis. Take in account that, if not highly diluted, the upper-phase samples migrate showing a lower molecular weight than expected (see section 3.5. Protein Analysis).
		Perform a Western Blot analysis with anti-LYTAG antibody (Cat No. AB-3238).
Elution of the fusion protein is inefficient	Choline concentration is limiting in the Elution solution.	Increase the choline chloride concentration in the Elution Solution from 300 mM to 500 mM (Step 6). Repeat the elution process if, after increasing the choline concentration, part of the protein is remaining in the upper phase.

5. Appendix

5.1. Related products

PRODUCT		DESCRIPTION	FORMAT	Cat.No.
KITS	LYTAG SpinPlus	New kit for recombinant expression and purification of protein in *E. coli*	Kit	KT-4662
	C-LYTAG protein expression & purification system	Kit for recombinant expression and purification kit of proteins in *E. coli*	Kit	KT-3246
	C-LYTAG Purification Kit	Kit for purifying recombinant protein expressed in *E. coli*.	Kit	KT-3412
VECTORS	pALEXa,b&c	Cloning and expression of C-LYTAG fusion proteins	8 µg	EV-3240/41/42
	pALEX2a,b&c	Cloning and expresion of LYTAG fusion proteins	8 µg	EV-4443/44/45
	pALEX2-Ca	Expression for the production of C-terminal fusion to C-LYTAG sequence	8 µg	EV-3435
STRAINS	*E. coli* REG-1	Propagation of pALEX vectors and constructs and protein expression	Stab	BS-3262
	E. coli REG-12	Propagation of pALEX2 vectors and constructs and protein expression	Stab	BS-3458
REAGENTS AND SOLUTIONS	TSS solution	Preparation of competent cells	1.5 ml	RS-3215
	Salicylate	Inducer	25 ml (1M)	RS-3247
	PROLYSE (1X)	Protein extraction solution	25 ml	RS-3399
	LYTRAP Resin	Resin for LYTAG protein purification	30 ml	RS-3302
	Choline Chloride	Elution agent	20 ml (3M)	RS-3287
ANTIBODY	Anti-LYTAG	Polyclonal antibody	100 µl	AB-3238
OTHERS	Multibind 96 C-LYTAG	Multi-well format for multiple assays and high throughput screenings of biomolecules tagged with C-LYTAG	1 plate 5 plates	RS-3317 RS-3318
	C-LYTRAP Spin columns	Microcentrifuge columns containing LYTRAP resin	10 units 15 units 100 units	RS-3319 RS-3320 RS-3321
	Purification column	Empty columns for LYTRAP packing	1 unit (reusable)	RS-3322
ANTIBIOTICS	Ampicillin	Selection antibiotic cell culture tested	5 g	RS-3217
	Kanamycin	Selection antibiotic cell culture tested	5 g	RS-3219

5.2. References

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2.- Sanz *et al* (1988). *FEBS Lett.* **232**, 308-312.
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6.- Royo *et al* (2005). *J Biotechnol.* **116**,113-24.

NOTES