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Low-cost recombinase polymerase amplification (RPA)

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This protocol describes the expression of enzymes and creation of a master mix for recombinase polymerase amplification (RPA) assays.

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Lysis buffer:
50mM NaPO4, 500mM NaCl, 20mM imidazole, 10% glycerol, 10mM Mg2Cl2, 3mM bME , pH 7.2
(To the extract protease inhibittor capsules)

to make 100ml of the buffer: 0.69g of $NaH_2PO_4.H_2O$, 2.92 g of NaCl, 10 ml glycerol (100%), 0.136 imidazole, 0.095 $MgCl_2$, 20ul of beta Mercaptoethanoal (14.3 M)

Loading/Binding buffer
50mM $NaPO_4$, 500mM NaCl, 20mM imidazole, pH 7.2
To make 200ml of the buffer: 1.38 of $NaH_2PO_4.H_2O$, 5.84g of NaCl, 0.272g imidazole
titrate with NaOH to adjust pH

Elution buffer
20mM Tris, 500mM NaCl, 500mM Imidazole, 3mM bME, pH 8.0 (for UvsY keep the pH at 7.5)
To make 100ml of the buffer: 0.242g of Tris, 2.92 g NaCl, 3.4 g imidazole, 20ul of bME (14.3 M)
To make 50ml of the buffer: 0.121g Tris, 1.46g NaCl, 1.7g imidazole, 10ul bME pH 7.5

Storage buffer:
Gp32:20mM Tris, 500mM NaCl,20% glycerol, 2mM DTT, pH 8.0
To make 200ml of this buffer: 0.49 g tris, 5.84g NaCl, 40ml glycerol, 0.0617g of DTT
UvsX:20mM Tris, 300mM NaCl, 1mM DTT
To make 200ml of this buffer: 0.49 g tris, 3.54g of NaCl, 0.03g of DTT
Bsu:20mM Tris, 500mM NaCl, 1mM DTT, 10% glycerol pH 8.0
UvsY:20mM Tris, 500mM NaCl, 1mM DTT, 10% glycerol pH 8.0
To make 500ml of this buffer: 1.21g Tris, 29.2g NaCl, 0.15g DTT, 50ml glycerol
adjust 250ml at pH 8.0 and remaining 250ml to pH 8.0

Reaction Mix(2x): 2mM DTT, 5% PEG-35K (PEG 20K is a better choice), 50mM Tris (pH 8.0), 100mM potassium acetate
To make 20ml of 2X Reaction Mix:
4mM DTT: add 12.3 mg of DTT
10% PEG-20K: add 2 g of the powder
100 mM Tris 8.0: add 0.242 g
200 mM Potassium acetate: add 0.393 g
Make up 20 ml with ddH20, adjust pH to 8.0
Make aliquots of 1-2 ml and freeze at -20C

Mg soln(20x): 14mM $MgCl_2$ (or Magnesium Sulfate)
to make 10 ml of 20x Magnesium chloride solution of 280 mM concentration
add 0.266g of magnesium chlordie in 10ml water

Energy Mix (10x):
3 mM ATP, 50 mM phosphocreatine
to make 5ml of 10x energy mix:
ATP dipotassium: 0.0875g
creatine phosphate disodium tetrahydrate: 0.81785g

Enzyme Mix(10x):
10x of enzyme mix will have
Bsu: 0.3ug/ul
UvsY: 1.2 ug/ul
UvsX: 0.6ug/ul
Gp32: 9ug/ul

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Creatine Kinase: 1 ug/ul

Culturing cells

1 1. Grow BL21 cells in LB media with appropriate antibiotics, overnight at **37 °C** with shaking (for long term, store the pellets in **-80 °C**).
2. Inoculate **1 mL** of the overnight culture to fresh **100 mL** LB (whatever capacity required) with right concentration of antibiotics and continue growing at **37 °C** whilst shaking.
3. At ~ OD600 of 0.5, induce the expression of the gene using **100 mL** IPTG and culture them at **15 °C** overnight, with continuous aeration.

Cell lysis 20s

2 1. The following day, centrifuge the culture by spinning down to maximum g force and then 20s resuspend in **10 mL** of 1X ice cold binding buffer. Add 25-40KU of lyzozyme per g of cells to break cell wall. Note: Benzonase addition can help break E.coli DNA. Novagen protocol suggests to avoid it as it could be in final purified protein. Hence, a suggestion is to avoid adding as it could possibly interfere in RPA and Cas 12 assays. note: addition of protease inhibitor is optional according to many protocols, add according to the manufacture's instructions.
2. Sonicate on ice for few alternating cycles (30 cycles) of sonication and hold for **00:00:20** each.
3. Centrifuge the lysate at maximum g force, collect the supernatant and 0.22 um filter syringe to get rid of traces of cell debris that could otherwise clog the column. If you have no 0.22um filter, repeat spinning down the supernatant again and use the supernatant for further steps.

Column purification 20s

3 1. Mix and load 6 ml of the resin (or any available Ni-NTA column) along with it's storage buffer and let the resin gravity settle. The millipore Ni-NTA agarose resin has 50% resin and the remaining 50% is loading buffer. So the column volume (CV) is 3ml in this case. Discard the flow through.
2. When the level of storage buffer reaches the top of the resin, equilibrate the column with following washes in sequence (i) 3 CV of MiliQ water (ii) 5 CV of 1X charge buffer. If your resin is precharged, skip this step (iii) 3 CV binding buffer.
3. Load the cell extract.
4. Wash the column with 10 CV of binding buffer.
5. Elute the bound protein through 10 CV of elute buffer, collect the eluate. Eluate can be

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stored in 4 °C until dialysed to storage buffer. The protein in eluate buffer is not stable for long in 4 °C or in the elution buffer, hence dialyse to storage buffer within a day or two of elution.

6. Strip the Ni from the column using the stripping buffer.
7. To re-use the resin same day , follow step 1 or Store column in 20% ethanol in 4 °C

Dialysis and protein concentration

4 Dialyse the eluate with it's respective storage buffer. Follow the video in this link for instructions.
concentrate the elaute using a concentrator to required concentration (if your protein working concentration is 600ng/ul, atleast concentrate to 12-20 ug/ul)

The quickest way to measure the concentration is by measuring absorbance at 280nm (A280) using a nano drop. The extinction coefficient for the protein of interest can be measured online using Extinction coefficient calculator. Note that this method although considers the extinction coefficient of protein of interest, A280 values read are of all the protein in the sample. Hence, measuring accuracy depends on the purity of the sample. Accurate concentration can be also quickly measured using semi-quantitative SDS page (contact Smitha for python based tools for this).

RPA protocol 1h 30m

5 1h 30m

1. Add the below component, first incubate at 37 °C for 00:30:00 to 01:00:00 (always add enzymes at the end).
2. Measure the fluorescence for Eva green (or any other dye like SYBER, SYTO can be used for real-time monitoring of RPA) with excitation at 495 nm and emission at 520 nm

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A	ul to add
template	add accordingly
water	add accordingly
Rxn Mix (2x)	5
Total volume	10
dNTP(10mM)	1.5
energy mix (10x)	1
Enzyme mix C (10x)	1
MgCl2 (280 mM)	0.5
FP (100uM)	0.1
RP(100uM)	0.1
eva green 100x	0.1

1. 2x Reaction mix works faster with a final pH above 8.0 as the isolectric point of UvsY is around 7.8. Having at pH 8.0 in reaction buffer reduces the appearances of cloudy precipitated protein while making RPA mix, which predominatly was noted as UvsY.
2. Certain combination of RPA primers work faster at 39-40C than 37C, so check the optimal temperature for the primer set
3. Purity of the RPA enzymes does affect the speed of the reaction. An anion chromatography step following the Ni-NTA purification results in faster RPA reaction.

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