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Derivitization of polyphosphate with cystamine to facilitate secondary labeling with biotin

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1 Works for me dx.doi.org/10.17504/protocols.io.7z3hp8n

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ABSTRACT

Investigation of the biological roles of inorganic polyphosphate has been facilitated by our previous development of a carbodiimide-based method for covalently coupling primary amine-containing molecules to the terminal phosphates of polyphosphate (Choi et al., Biochemistry 49:9935, 2010). We now extend that approach by using readily available “bridging molecules” containing a primary amine and an additional reactive moiety, including another primary amine, a thiol or a click chemistry reagent such as dibenzocyclooctyne. This two-step labeling method is used to covalently attach commercially available derivatives of biotin, peptide epitope tags, and fluorescent dyes to the ends of polyphosphate. This protocol specifically describes the labeling of heterogenous long-chain polyP with cystamine and the subsequent addition of maleimide-biotin.

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GUIDELINES

PolyP concentration is measured and described in terms of phosphate monomer concentration. To determine the concentration of ends, multiply the monomer concentration by two, then divide by the mode chain length.

MATERIALS

NAME	CATALOG #	VENDOR
Polyphosphate Long Chain (p700)	EUI002	Kerafast
N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC)	E1769	Sigma Aldrich
3-(N-Morpholino)propanesulfonic acid 4-Morpholinepropanesulfonic acid (MOPS)	M1254	Millipore Sigma
Cystamine dihydrochloride	30050	Millipore Sigma
Zeba™ Spin Desalting Columns 7K MWCO 5 mL	89891	Thermo Fisher Scientific
TCEP	20490	Fisher Scientific
EZ-Link maleimide-PEG2-biotin	PI21901	Thermo Fisher Scientific
HEPES	H3375	Sigma Aldrich
EDTA	EDS	Sigma Aldrich

1 Prepare and combine all materials **in this order** to a 1.5, 2 or 5 mL tube.

	Volume (µL)	Final Concentration
Water	659	
500 mM MOPS pH 8.0	200	100mM
1M polyP (mode chain length: 1000)	50	100 µM ends (50 mM phosphate monomer)
500 mM Cystamine	16	8 mM (80x ends)
2M EDAC	75	150mM
total	1000	

At the last minute, dissolve dry EDAC powder in water to make a 2 M solution. Then add to reaction.

NOTE: EDAC is very hydroscopic and will degrade with exposure to humidity. Store powder aliquots of EDAC at -20 °C, dessicated. For each reaction, remove aliquot from freezer, allow to warm to room temperature. Refrain from dissolving until immediately before use. Do not store dissolved EDAC.

2 Immediately incubate at 37 °C for 1 h, agitating occasionally.

3 Stop reaction on ice.

4 Centrifuge briefly to remove any condensation from the top of the tube.

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5 Follow manufacturer's instructions for 5 mL Zeba™ Spin Desalting Columns (7K MWCO), exchanging the buffer into 20 mM Hepes-NaOH pH 7.4 + 5 mM EDTA to remove excess cystamine and to prepare for the upcoming maleimide reaction.

6 Add TCEP to a final concentration of 5 mM (dissolve 5.5 mg TCEP in 191 µL water to make 100mM solution, then add 50 µL to 1000 µL of polyP reaction solution).

7 Rotate the tube at room temperature for 1.5 h.

8 Follow manufacturer's instructions for 5 mL Zeba™ Spin Desalting Columns (7K MWCO), exchanging the buffer into 20 mM Hepes-NaOH pH 7.4 + 5 mM EDTA to remove TCEP.

9 Dissolve 6.5 mg maleimide-biotin in 50 uL water to make a 250 mM solution.

Combine 32 µL of the 250 mM maleimide-biotin solution to 1000 µL of purified, reduced polyP-cystamine sample to make a 8 mM maleimide biotin solution.

10 Vortex briefly then rotate at room temperature for 1.5 h.

11 Follow manufacturer's instructions for 5 mL Zeba™ Spin Desalting Columns (7K MWCO), exchanging the buffer into 20 mM Hepes-NaOH pH 7.4 + 5 mM EDTA to remove excess biotin.

Note: this buffer can be replaced depending on the requirements of future experiments. However, for EDAC-labeled polyP we suggest storage in a buffer with a pH between 7 and 8 and at least 100 µM EDTA to chelate trace metals.

12 Remove an aliquot to quantify the phosphate using malachite green quantification assay using standards diluted in the same buffer used in the previous step.

Zhou X, Arthur G. (1992). Improved procedures for the determination of lipid phosphorus by malachite green.. J Lipid Res..

See the paper

13 If desired, quantify the concentration of biotin using the Pierce Fluorescence Biotin Quantitation Kit according to the manufacturer's instructions.

14 Using the results of the malachite green and biotin quantitation, we can get a rough estimate of the degree of labeling

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(concentration of monophosphate from malachite green)/(mode polymer length)= concentration of polymer

(concentration of polymer) *2 = concentration of polyphosphate ends

(concentration of biotin)/(concentration of ends) = ratio of biotin to ends

NOTE: because polyP is heterogeneous, this calculation is based on the mode chain length, and is merely an approximation.

It's not uncommon that your ratio of biotin to ends might be >1. Polymers chains shorter than the mode can cause this. However, if the ratio is way higher (>10, for example) it may be worth confirming the purification step to remove excess reactant biotin is working properly (Step 11) .

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