# Protein Science - Nucleotide Exchange of KRAS4b

## Purpose

This protocol covers the steps we use for exchanging the resident nucleotide in samples of KRAS4b to the non-hydrolysable nucleotide GppNHp. It is likely also applicable for other closely related G-proteins.

## Scope

This protocol details the nucleotide exchange KRAS4b (with some comments that may be relevant to different forms of the protein; e.g., FMe or just the G domain). In the example provided, the amount of the starting protein is 11 mg, which is typically the largest scale we perform in a single reaction. If additional material is needed, we create replicates of this scale. The extent of nucleotide exchange can be assessed by HPLC, but is not covered in this protocol.

## Definitions

**GDP:** guanosine diphosphate

**GppNHp:** guanosine-5′-[(β,γ)-imido]triphosphate

**MWCO:** molecular weight cut-off

**TCEP:** tris(2-carboxyethyl)phosphine

## Materials and Equipment

- 1.5 mL tubes (USA Scientific, Inc., 1615-5500)
- Phosphatase, Alkaline–Agarose, agarose beads (MilliporeSigma, cat# P0762)
- PD10 desalting columns (Cytiva, 17085101)
- 10,000 MWCO centrifugal concentrator (if desired)

## Safety Precautions

Use standard laboratory personal protective equipment.

## Procedures

A. Protein Preparation

The $MgCl_2$ concentration must be kept below 1 mM to ensure an efficient exchange as it plays a role in bound nucleotide stability. Thus, a buffer exchange and re-assay of the protein concentration may be necessary.

The protein will be diluted with addition of components and thus a convenient final volume is chosen that might have more to do with downstream application convenience that the required parameters of the exchange. Typically the protein concentration in the exchange is ~10–250 µM.

B. Reaction Setup (3 mL Reaction for a 2 mL 258 µM Starting Protein Sample)

1. Thaw the protein on ice.
2. Add the following to the 2 mL protein sample (see special note about the addition of the last component, ammonium sulfate):
   a. 103 µL of 50 mM GppNHp
   b. 3 µL of 0.1 M $ZnCl_2$
   c. 594 µL of the protein buffer (for KRAS G-domain: 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM TCEP)
   d. 300 µL of 2 M ammonium sulfate (Note: it is important to add this component last and mix this quickly while adding to avoid localized areas of high ammonium sulfate concentration).

Final reaction conditions:

- 172 µM protein
- 1.72 mM GppNHp (or 10× the protein concentration)
- mM $ZnCl_2$
- 200 mM ammonium sulfate

C. Alkaline Phosphatase Addition

1. Add beads at 1 U/mg of protein (11 units in this example).
2. The alkaline phosphatase concentration is 0.25 U/mL of resin, but the resin comes diluted as a 50% slurry, thus 88 µL would be required for 11 units (11/0.25 × 2). Add 88 µL of beads slurry to the reaction and mix gently at room temperature for 2–3 hours at ~21°C.

   Note: Alkaline phosphatase will remove phosphates from any form of hydrolysable nucleotide removed from the protein, thus allowing the non-hydrolyzable nucleotide to be bound by the protein.

**D.** Stabilize Protein

1. Spin out the beads and remove the supernatant to a fresh tube.
2. Adjust the sample to 5 mM $MgCl_2$.
3. Add an additional aliquot of 10× GppNHp (here, 103 µL).
4. Mix well and incubate (no shaking required) at room temperature for 1–2 hours or overnight at 4°C.

**E.** Buffer Exchange and Final Sample Assay and Storage

1. Remove excess GppNHp via gel filtration (PD10 column) into your preferred final buffer. If using a protein that is the post-translationally modified farnesylated, methylated KRAS, the final buffer should have 300 mM NaCl for stability.
2. The final protein is typically assayed for protein concentration, concentrated, or diluted as necessary for final storage, dispensed in $\leq$ 250 µL aliquots in 1.5 mL tubes, snap-frozen in liquid nitrogen, and stored at –80°C.