Basic INSTRUCTIONS FOR USE

***Components provided*:**

- BirA biotin-protein ligase (1mg/ml [BirA500] or 3mg/ml [Bulk BirA])
- BiomixA (10x concentration: 0.5M bicine buffer, pH 8.3)
- BiomixB (10x concentration: 100mM ATP, 100mM MgOAc, 500µM d-biotin)
- BIO200 (10x concentration: 500µM d-biotin)

The final reaction will contain the following:

- 1/10 BiomixA (other buffers may be used, see below)
- 1/10 BiomixB
- 8/10 dH_2O – substrate volume
- Substrate peptide containing AviTag™ (for concentration see discussion below)
- BirA enzyme (2 µg/100 µg of substrate is a good starting point)

  For example, a typical 1 mL reaction mix might look like this:

  - 700 µL dH_2O
  - 100 µL BiomixA
  - 100 µL BiomixB
  - 100 µL AviTag'd substrate (100 µg at 1 mg/mL)
  - 2 µg BirA (2 µL at 1 mg/mL or 0.7 µL at 3 mg/mL)

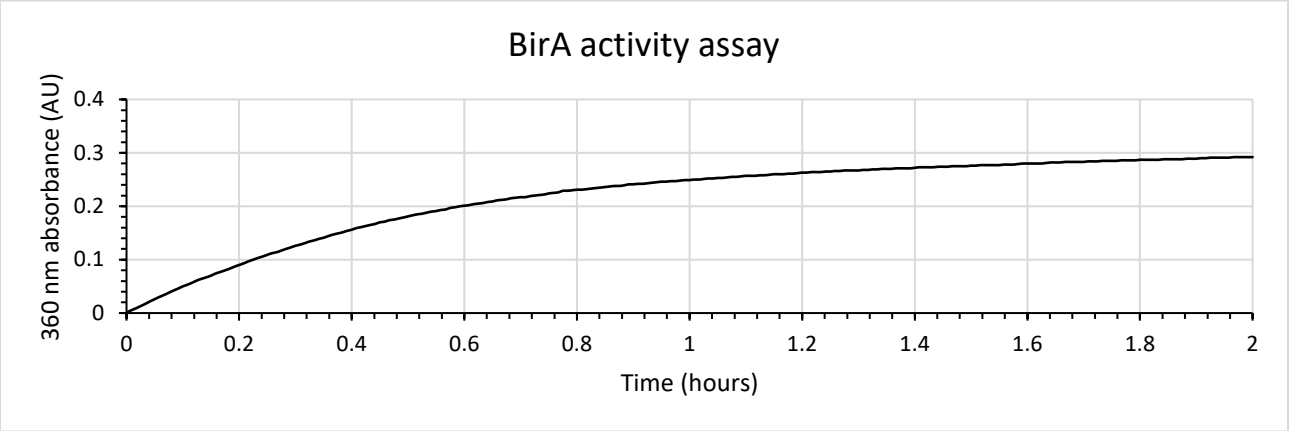
Ensure that the reaction proceeds for a minimum of one hour at room temperature, as it typically achieves a completion rate of ≥85% within this timeframe. Extending the duration to over two hours may be required for full completion, as the reaction rate decreases as components are depleted. Increasing the amount of BirA enzyme can expedite the reaction, provided that all essential components are available. These reaction conditions serve as general guidelines that can be used as a starting point to determine the optimal conditions for your specific requirements.

Buffer conditions that affect biotin ligase:

It is important to be aware that certain reagents commonly found in biological buffers can reduce the activity of BirA enzyme. These include NaCl (specifically Cl^-), glycerol, and ammonium sulfate, among others. As a result, the concentration of these reagents in the substrate solution should be minimized. We recommend suspending the substrate in 50 mM bicine, pH 8.3 (see BiomixA), although other buffers may be substituted. If the protein substrate requires high salt conditions for stability or activity, consider substituting potassium glutamate for sodium chloride. Potassium glutamate is compatible with biotin ligase at higher concentrations and can provide an ionic environment that stabilizes proteins.

BiomixA is a bicine reaction buffer, pH 8.3, which we use and recommend in most cases because it works well with BirA. However, BirA will also function in various other buffer conditions, so you are not limited to this one. While we cannot predict all buffer conditions that will favor your substrate, you can expect good biotinylation results with BirA in most cases.

BiomixB contains ATP, which may be subject to hydrolysis if not handled correctly. As the most labile component of the BirA reaction kit, BiomixB should be thawed on ice and immediately refrozen after use. Prolonged incubation at room temperature can lead to ATP hydrolysis, reducing the available ATP for the biotinylation reaction. Completing the reaction quickly will help achieve maximal biotinylation.



The conditions illustrated in the graph above show the rate-of-reaction for a particular 16-amino acid substrate (the actual AviTag sequence with a C-terminal cysteine) that we use to measure BirA activity in-house. These conditions have been fine-tuned for rapid, reproducible biotinylation of this particular peptide and form the basis of our reaction protocol. However, it is important to note that the optimal conditions may vary for other AviTag'd substrates. Nevertheless, these general conditions should yield satisfactory biotinylation levels.

To achieve maximum biotinylation in the shortest possible time, you may need to adjust the reaction mix to suit your substrate. BirA operates effectively across a wide range of pH conditions, substrate concentrations, buffer conditions, reaction temperatures, etc. This flexibility is a key advantage of our BirA enzyme, which can function even in less-than-ideal conditions. It is also worth noting that 100% biotinylation is an idealized goal that may not be achievable within a reasonable timeframe. Typically, 95% biotinylation of the available substrate is considered complete.

Reaction details:

Proceed with the above information to begin your reactions. However, if you wish to fine-tune the reaction to include more precise BirA quantities for reproducibility or quality control, perhaps in using different AviTag'd substrates, please read on.

Our BirA reaction is developed around substrates at 40 µM in the reaction mix. The components in BiomixB, specifically d-biotin and ATP, are designed to work in solutions with a 40 µM substrate concentration. Therefore, it is recommended that your AviTag substrate be close to 40 µM in the final reaction mix to match the available components. Lower substrate concentrations will require longer to complete biotinylation and more BirA enzyme for comparable rates. For example, a substrate at 40 µM may be fully biotinylated within 30-40 minutes, but at 4 µM, it could take over 5 hours using the same amount of BirA enzyme. Even at 40 µM, the amount of BirA enzyme added to the reaction mix may need to be adjusted to achieve biotinylation within an acceptable timeframe due to the availability of the AviTag on your substrate's tertiary construct. If biotinylating substrates at concentrations up to 80 µM, supplementing the reaction mix with additional BirA and biotin (included) may be necessary. For concentrations above 80 µM, please contact our Technical Support at info@avidity.com or call Toll-Free at (877) 333-6063. Emails are preferred for communication.

Typically, biotinylation for 30-40 minutes at 30°C or 1 hour at room temperature will result in at least 85% completion. Reactions can also be performed at 4°C but will require more time, often overnight. The Avidity BirA is stable at 4°C and will continue biotinylation for extended periods if there is adequate substrate and

d-biotin available. Our enzyme is tested and shown to be free from measurable protease contamination, and endotoxin levels are below the Endosafe PTS detection limit of 0.05 EU/mL. However, longer incubation times could increase the risk of target protein degradation from non-kit contaminants. Therefore, we recommend using the shortest incubation time that fully biotinylates the target protein.

Example calculations that we use in lab for determining the amount of BirA needed for reactions:

To precisely calculate the parameters of your specific BirA reaction or to understand our methodology, we provide example calculations using an imaginary substrate containing the AviTag peptide sequence. The total molecular weight is 15 kDa (note: the 15aa AviTag has a molecular weight of 1.829 kDa). Two methods can calculate the required amount of BirA, both based on substrate concentrations in nmol and 2 µg of BirA per 10 nmol (instead of µg) of AviTag'd substrate.

By microgram calculation:

- 1 Da = 1 g/mol; 15 kDa = 15,000 g/mol; 1 mol/L = 1M = 15,000 g/L (15,000 mg/mL); 1 mg/mL = 0.0000667 M = 66.7 µM. Our target substrate concentration for this reaction is 40 µM, so our substrate needs to be diluted 6/10 in our reaction mix. (1 mg/mL)(0.6 mL) = 0.6 mg (600 µg).
- 1 nmol = 15 µg; 10 nmol = 150 µg. 600 µg is 40 nmol. 2 ug of BirA /10 nmol of substrate = 8 µg BirA*.

By nmol calculation:

- 15 kDa protein at 1mg/mL = 1000 µg/mL = 66.7 nmol/mL = 66.7 µmol/L = 66.7 µM. Our target concentration for this reaction is 40 µM. We need to dilute the reaction solution to 40 µM final, or 6 parts in 10 (600 µL/1 mL reaction).
- 40 µM = 40 µmol/L = 40 nmol/mL; 2 µg BirA/10 nmol substrate = 8 µg BirA*.

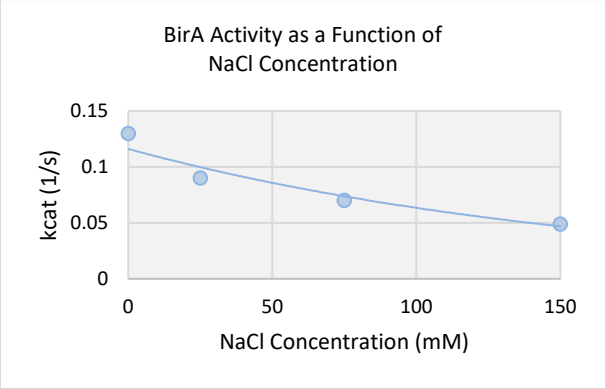
So, the final 1 mL reaction mix would look like this:

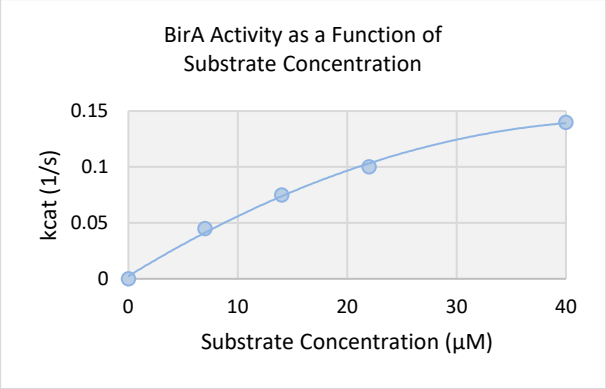
- 600 µL of 1 mg/mL substrate
- 100 µL BiomixA
- 100 µL BiomixB
- 200 µL dH_2O
- 8 µg BirA (8 µL of 1 mg/mL or 2.7 µL of 3 mg/mL)

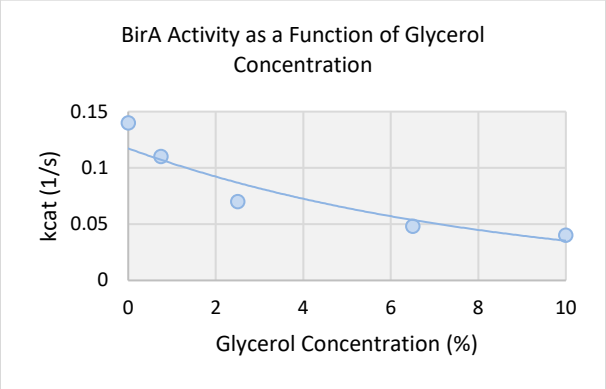
*Notice that in our initial guidelines for BirA to substrate ratio we recommend 2 µg BirA /100 µg of substrate for starters. But by this estimation the amount of BirA added to this reaction should be 12 µg, not 8 µg. Why the difference? We arrived at our starting guidelines purely for the sake of simplicity, to get a researcher new to our technology a good place to start, and 2 µg BirA /100 µg substrate will get most reactions to completion in an acceptable time frame. The example calculations are to aid in arriving at BirA quantities more in line with our in-house methods. These methods may or may not better suit your requirements. They are instead for normalizing BirA reaction rates for product quality control here at Avidity and may help you in establishing your own reaction rate standards, if required.

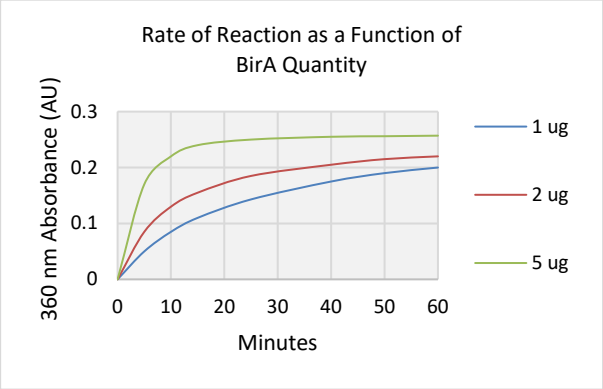
But, when in doubt, more BirA never hurts and will always increase reaction speed.

Appendices:

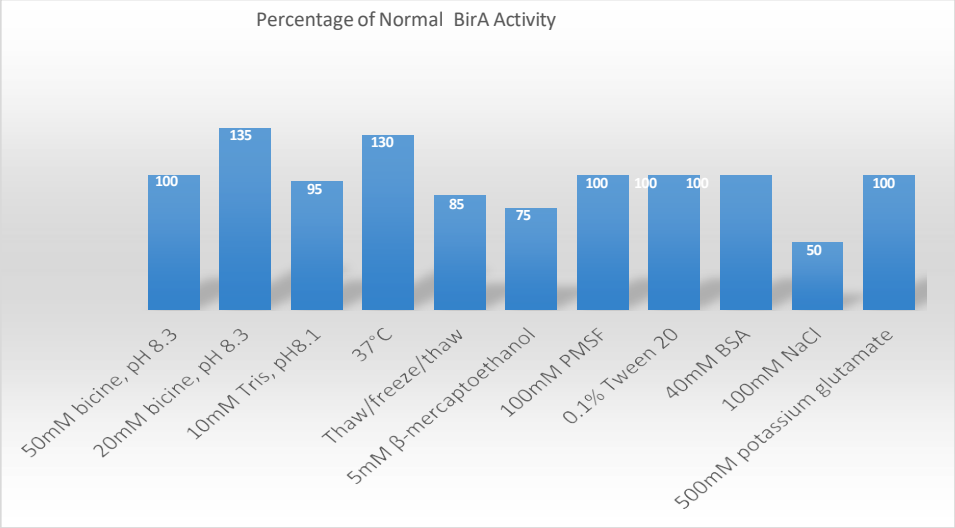








Levels of biotin ligase (BirA) activity in various buffer conditions



Related products:

BIS-300 Positive Control Substrate Kit:
The BIS-300 Kit contains a fully biotinylated MBP-AviTag™ fusion protein standard and an unbiotinylated MBP-AviTag™ fusion protein that may be used as an extent-of-biotinylation comparison for BirA biotin- protein ligase reactions, for SDS-PAGE gel analysis or in Western blot analysis.