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HPG/AHA Protein Synthesis Assay Protocol Fluorescent Microscopy

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L-homopropargylglycine (HPG) and L -Azidohomoalanine (AHA) are a non-radioactive alternative to the traditional ^{35}S-methionine which is incorporated into proteins during active protein synthesis and can be directly added to cells. Commercial HPG- and AHA-based kits used for detecting of de novo synthesized proteins provide great results, but are often quite expensive and provide fixed amounts of reagents, which limits optimized or off-protocol use of these kits. Self-assembled kits are a viable alternative to commercially available kits, in particular when all of the components are widely available from a number of suppliers. The amounts of reagents and the click reaction conditions are very similar between many commercial kits, and are in line with large number of published procedures for HPG- and AHA-based detection of newly synthesized proteins. Using the provided protocols, a researcher will be able to assemble an HPG or AHA Protein Synthesis Assay that would require very little, if any, fine tuning.

These kits with improved biocompatibility and detection limits were first commercialized by ThermoFisher Scientific and sold under Click-iT® HPG and Click-iT® AHA. Click Chemistry Tools kits take advantage of next generation, copper chelating azides. The introduction of a copper-chelating moiety at the azide reporter molecule allows for a dramatic increase of the effective Cu(I) concentration at the reaction site, enhancing the weakest link in the reaction rate acceleration, greatly increasing the sensitivity and biocompatibility of HPG- and AHA-based assays for analyzing protein synthesis in cells.

Materials Required

- HPG (L-Homopropargylglycine, https://clickchemistrytools.com/product/l-homopropargylglycine-hpg/) or AHA (L-Azidohomoalanine, https://clickchemistrytools.com/product/l-azidohomoalanine-aha/)
- AFDye Picolyl Azide (https://clickchemistrytools.com/product-category/azides-2/fluorescent-picolyl-azides/)
- Copper (II) Sulfate pentahydrate https://www.sigmaaldrich.com/catalog/product/sigald/c7631?lang=en&region=US
- THPTA (https://clickchemistrytools.com/product/thpta/)
- Sodium ascorbate (https://www.sigmaaldrich.com/catalog/product/sigma/a4034?lang=en&region=US)
- Fixative (3.7% formaldehyde in PBS)
- Permeabilization reagent (for example, 0.5% solution of Triton®X-100 in PBS)
- 3% BSA in PBS (pH 7.4),
- Hoechst 33342 (optional)

Material Preparation

- **HPG/AHA Stock Solution:** Prepare 50 mM solution of HPG or AHA in DMSO or water, for example to make 1 mL of 50 mM stock solution dissolve 8 mg of HPG or 9 mg of AHA in 1 mL of DMSO or water.
- **AFDye Picolyl Azide Stock Solution:** Prepare 1 mM solution in DMSO or water. Example: to make 150 µL dissolve the entire AFDye Picolyl Azide Kit Pack in 150 µL of DMSO or water.
- **Copper Catalyst (25 mM $CuSO_4$, 62.5 mM THPTA) solution:** Weight out 312 mg of Copper (II) Sulfate Pentahydrate and 1.35 g of THPTA, mix, add 50 mL of water, vortex to dissolve completely.
- **Reaction Buffer:** 50 mM Tris, 150 mM NaCl, pH 7.5. Dissolve 3.02 g of Tris, 4.4 g of NaCl in 500 mL of water, adjust pH to 7.5, sterile filter.
- **Hoechst 33342:** 10 mg/mL stock solution. Dissolve 1 mg of Hoechst 33342 in 100 µL of DI water.
- **Reducing Agent:** Dissolve 20 mg of Sodium Ascorbate in 1.8 mL of deionized water. Vortex until completely dissolved. Sodium ascorbate solution is susceptible to oxidation. We recommend always using freshly prepared solution of sodium ascorbate.
- **Wash buffer** (0.5 mM EDTA, 2 mM NaN_3 in PBS): Add 1 mL of 0.5 M EDTA and 0.13 g of dry sodium azide to 1 L of PBS. Sterile filter for long term storage.

Cell labeling with HPG/AHA

This protocol is based on a large number of publications of HPG- and AHA-based procedures for analyzing peptide synthesis in cells used with different types of cells. An optimized HPG/AHA concentration is 50 µM but may need adjustment depending on the given cell type. Growth medium, cell density, cell type variations, and other factors may influence

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labeling. Investigators are encouraged to determine the optimal concentration of the HPG reagent as well as labeling time individually for each cell type on a small-scale first.

1.1 Plate the cells on coverslips at the desired density and allow them to recover overnight before additional treatment.

1.2 Prepare 50 mM solution of HPG or AHA in DMSO or water.

1.3 Wash cells once with PBS, add methionine-free medium and incubate the cells at 37°C for 30–60 minutes to deplete methionine reserves.

1.4 Add desired amount of HPG or AHA to cells in L-methionine-free culture medium to achieve optimal working HPG/AHA concentration (50 µM, if not optimized).

1.5 During addition of HPG or AHA to cells in culture, avoid disturbing the cells in ways that may disrupt the normal cell cycling patterns.

1.6 Incubate the cells for the desired length of time under conditions optimal for the cell type. Different cell types may require different incubation periods for optimal labeling with HPG or AHA. As a starting point we recommend 50 µM HPG or AHA for 1 hour.

1.7 Proceed immediately to **Cell fixation and permeabilization.**

Cell fixation and permeabilization

The following protocol is provided for the fixation step using 3.7% formaldehyde in PBS followed by a 0.5% Triton®X-100 permeabilization step. Protocols using other fixation/permeabilization reagents, such as methanol and saponin, can also be used.

2.1 Transfer each coverslip into a single well. For convenient processing, use 6-well plates.

2.2 After metabolic labeling, remove the media and add 1 mL of 3.7% formaldehyde in PBS to each well containing the coverslips. Incubate for 15 minutes at room temperature.

2.3 Remove the fixative and wash the cells in each well twice with 1 mL of 3% BSA in PBS.

2.4 Remove the wash solution. Add 1 mL of 0.5% Triton® X-100 in PBS to each well, then incubate at room temperature for 20 minutes.

HPG/AHA detection

Note: 500 µL of the reaction cocktail is used per coverslip. A smaller volume can be used as long as the remaining reaction components are maintained at the same ratios.

3.1 Prepare the required amount of the reaction cocktail according to Table 1. **Add the ingredients in the order listed in the table.** Use the reaction cocktail within 15 minutes of preparation.

Table 1.

Reaction component	Number of coverslips						
	1	2	4	5	10	25	50
1x Reaction Buffer (Material preparation)	430 µL	860 µL	1.7 mL	2.2 mL	4.3 mL	10.7 mL	21.4 mL
Copper Catalyst (Material preparation)	20 µL	40 µL	80 µL	100 µL	200 µL	500 µL	1 mL
Picolyl Azide Solution (Material preparation)	2.5 µL	5 µL	10 µL	12.5 µL	25 µL	62.5 µL	125 µL
Reducing Agent (Material preparation)	50 µL	100 µL	200 µL	250 µL	500 µL	1.25 mL	2.5 mL
Total Volume	500 µL	1 mL	2.0 mL	2.5 mL	5.0 mL	12.5 mL	25 mL

3.2 Remove the permeabilization buffer (step 2.4)
Wash the cells in each well twice with 1 mL of 3% BSA in PBS.
Remove the wash solution.

3.3 Add 0.5 mL of the **Reaction Cocktail** to each well containing a coverslip.
Rock the plate briefly to ensure that the reaction cocktail is distributed evenly over the coverslip.

3.4 **Protect from light,** and incubate the plate for 30 minutes at room temperature.

3.5 Remove the reaction cocktail.
Wash each well once with 1 mL of 3% BSA in PBS.
Remove the wash solution.

3.6 Wash each well once with 1 mL of **Wash Buffer.**
Remove the wash solution.

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3.7 Wash each well once with 1 mL of PBS.

Remove the wash solution.

At this point the samples are ready for **DNA staining**. If no **DNA staining** is desired, proceed to **Imaging.**

If antibody labeling of the samples is desired, proceed to labeling according to manufacturer's recommendations. **Keep the samples protected from light during incubation.**

DNA staining

4.1 Wash each well with 1 mL of PBS.
Remove the wash solution.

4.2 Prepare 1x **Hoechst 33342 solution** by diluting stock solution of **Hoechst 33342** 1:2,000. The final concentration of 1x **Hoechst 33342 solution** is 5 µg/mL.
Final concentrations of 1x **Hoechst 33342** may range from 2 µg/mL to 10 µg/mL.

4.3 Add 1 mL of 1x **Hoechst 33342 solution** per well. **Protected from light.** Incubate for 30 minutes at room temperature.

4.4 Remove the Hoechst 33342 solution.

4.5 Wash each well twice with 1 mL of PBS.

4.6 Remove the wash solution.

Imaging

Labeled cells are compatible with all methods of slide preparation.

References

1. Dieterich, D. C., Link, A. J., Graumann, J., Tirrell, D. A., & Schuman, E. M. (2006). Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT). *Proceedings of the National Academy of Sciences of the United States of America, 103*(25), 9482–9487. https://doi.org/10.1073/pnas.0601637103

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