Click-iT® Metabolic Labeling Reagents for Proteins

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Alkyne- or azide-tagged compound	Varies, see product label	• ≤–20˚C • Desiccate • Protect from light	When stored as directed, the product is stable for at least 6–12 months.

Introduction

Click chemistry describes a powerful new class of chemical reactions that use bio-orthogonal or biologically unique moieties to label and detect proteins of interest using a simple, two-step procedure.[1-4] In the first step, an azide- or alkyne-containing biomolecule is actively incorporated into the protein (Table 2). Unlike other labels, the azide- and alkyne-tag is small enough that tagged biomolecules (e.g., sugars[5] and amino acids[6]) are acceptable substrates for the enzymes that incorporate these building blocks into biopolymers such as proteins (Figures 1–2, Table 2). The second step, detection step, uses the chemoselective ligation or "click" reaction between an azide and an alkyne. In the click reaction, the modified protein is detected with a corresponding azide- or alkyne-containing dye or hapten (Figure 3, Table 3). Proteins labeled with a Click-iT® metabolic labeling reagent can be detected using the Click-iT® Cell Reaction Buffer Kit (Cat. no. C10269) for subsequent analysis by flow cytometry or imaging, or the Click-iT® Protein Reaction Buffer Kit (Cat. no. C10276) for subsequent analysis by standard biochemistry techniques such as gel electrophoresis.

Click chemistry can be used when methods such as direct labeling or the use of antibodies are not applicable or efficient. For deeper biological insight, prior to or directly after the click reaction, cells or tissue can be stained with additional detection reagents. Detection sensitivity in one dimensional (1D) gels and western blots has been found to be in the low femtomole range and compatible with downstream LC-MS/MS and MALDI-MS analysis.

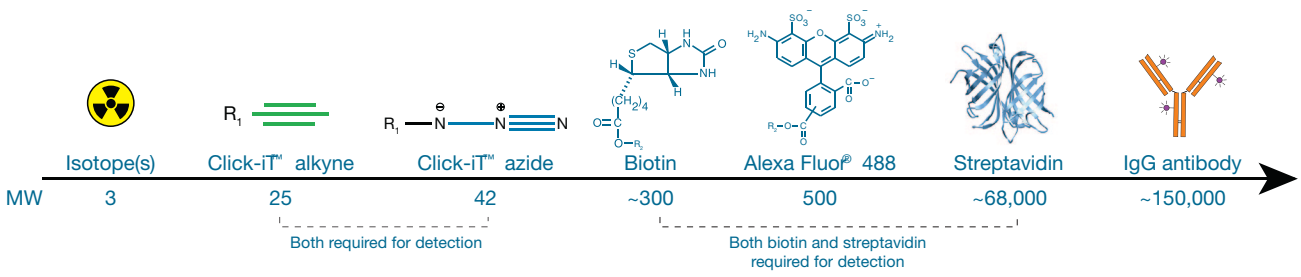

Figure 1. Relative size of detection molecules commonly used in cellular analysis.

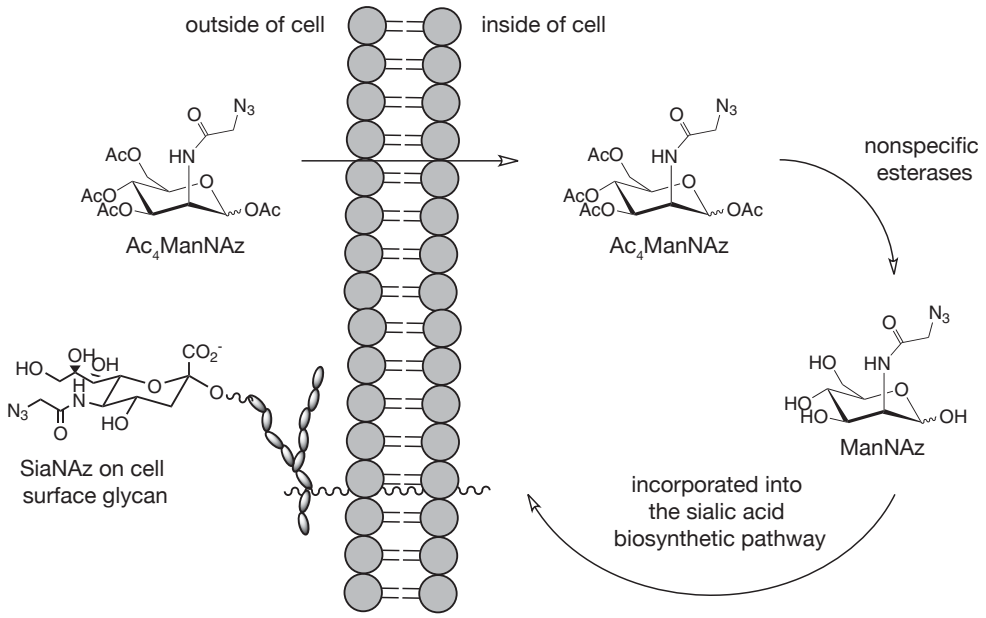

Figure 2. Metabolic incorporation of tetraacetylated azido-mannosamine (Ac_4ManNAz).

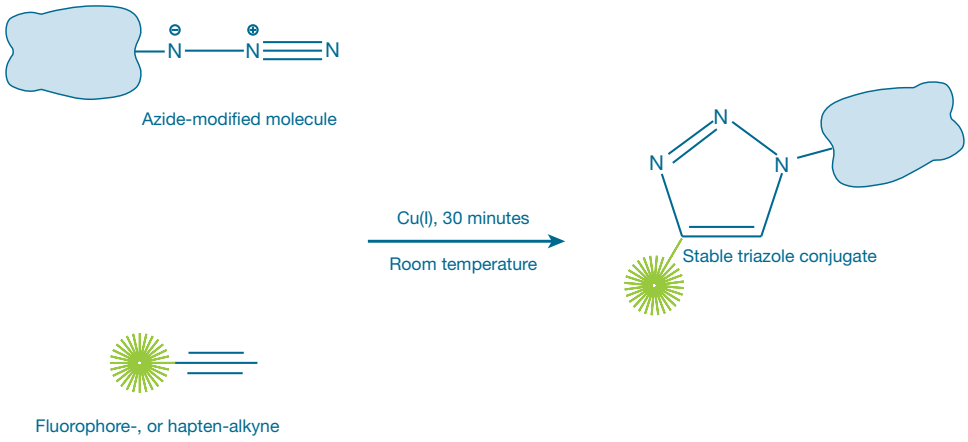

Figure 3. Click azide/alkyne reaction. The azide and alkyne moieties are interchangeable, whereupon the molecule can be labeled with an alkyne and react with a fluorophore- or hapten-azide.

Table 2. Click-iT® metabolic labeling reagents for proteins.

Compound	Cat. no.	Molecular weight	Application	Dose	Incubation time and conditions*
Click-iT® AHA (L-azidohomoalanine)**	C10102	258.16	Nascent protein synthesis	25–50 µM	1–4 hours; add to media when cells are 80–90% confluent. **Requires methionine-free media.**
Click-iT® HPG (L-homopropargylglycine)†	C10186	127.14			
Click-iT® farnesyl alcohol, azide**	C10248	263.38	Farnesylated proteins		24–48 hours; add to media when cells are 40–50% confluent.
Click-iT® geranylgeranyl alcohol, azide**	C10249	331.50	Geranylgeranylated proteins		
Click-iT® fucose alkyne (tetraacetylfucose alkyne)†	C10264	342.30	Fucosylated glycans		24–72 hours.
Click-iT® palmitic acid, azide**	C10265	283.41	Palmitoylated proteins	25–200 µM	4–6 hours.
Click-iT® myristic acid, azide**	C10268	241.33	Myristoylated proteins	10–50 µM	
Click-iT® GalNAz (tetraacetylated *N*-azidoacetylgalactosamine)**	C33365	430.37	*O*-Linked glycoproteins	25–50 µM	24–72 hours.
Click-iT® ManNAz (tetraacetylated *N*-azidoacetyl-D-mannosamine)**	C33366	430.37	Sialic acid-modified glycoproteins		
Click-iT® GlcNAz (tetraacetylated *N*-azidoacetylglucosamine)**	C33367	430.37	*O*-GlcNAz-modified glycoproteins		

*These are suggested doses and incubation times for typical immortalized cell lines (e.g., growth medium, cell density, cell type variations, and other factors may influence labeling). For initial experiments, we recommend testing a range of Click-iT® metabolic labeling reagent concentrations to determine the optimal concentration for your cell type and experimental conditions. Generally, reagents requiring labeling over 1 or more days may be added when the cells are seeded; however, some slow-growing cells may need time to become established prior to exposure to the labeling reagent.

**Compound contains an azide and requires an alkyne-containing molecule for detection.

†Compound contains an alkyne, and requires an azide-containing molecule for detection.

Table 3. Click-iT® detection reagents.

Detection reagent*	Azide or alkyne	Cat. no.	Ex/Em**	Use	Detection technique
Alexa Fluor® 488	azide	A10266	495/519	Fluorescent dye or hapten	• Flow cytometry • High content analysis (HCS) • Fluorescence microscopy
	alkyne	A10267			
Alexa Fluor® 555	azide	A20012	555/565	Fluorescent dye	• Fluorescence microscopy • High content analysis
	alkyne	A20013			
Alexa Fluor® 594	azide	A10270	590/617	Fluorescent dye	• Fluorescence microscopy • High content analysis
	alkyne	A10275			
Alexa Fluor® 647	azide	A10277	650/655	Fluorescent dye	• Flow cytometry • High content analysis • Fluorescence microscopy
	alkyne	A10278			
Biotin	azide	B10184	Not applicable	Hapten	• Flow cytometry† • High content analysis† • Fluorescence microscopy† • Western blot† • Mass spectrometry
	alkyne	B10185			
Oregon Green® 488	azide	O10180	496/524	Fluorescent dye or hapten	• Flow cytometry • High content analysis • Fluorescence microscopy
	alkyne	O10181			
Tetramethylrhodamine	azide	T10182	555/580	Fluorescent dye or hapten	• 1D or 2D gel electrophoresis • Western blot with an anti-TAMRA antibody • Mass spectrometry • Fluorescence microscopy • High content analysis
	alkyne	T10183			

*Use with Click-iT® Cell Reaction Buffer Kit (Cat. no. C10269) for subsequent analysis by flow cytometry or imaging, or Click-iT® Protein Reaction Buffer Kit (Cat. no. C10276) for subsequent analysis by standard biochemistry techniques such as gel electrophoresis.

**Approximate excitation/emission maxima in nm.

†Requires streptavidin conjugate.

Table 4. References for protein labeling and detection with click chemistry.

Topic	References
Azide and alkyne sugars for labeling glycoproteins	Luchansky, S. J., et al, (2003), Constructing azide-labeled cell surfaces using polysaccharide biosynthetic pathways, Methods Enzymol 362, 249. Hsu, T. L., et al, (2007), Alkynyl sugar analogs for the labeling and visualization of glycoconjugates in cells, Proc Natl Acad Sci 104, 2614.
AHA or HPG for nascent protein synthesis	Dieterich, D. C., et al, (2006), Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT), Proc Natl Acad Sci 103, 9482.
Prenylation	Chan Kim, S., et al, (2006), A tagging-via-substrate technology for genome-wide detection and identification of farnesylated proteins , Methods Enzymol 407, 629.
Fatty acylation	Hang, H. C. et al, (2007), Chemical Probes for the Rapid Detection of Fatty-Acylated Proteins in Mammalian Cells , J Am Chem Soc 129, 2744.
Fucosylation	Hsu, T. L., et al, (2007), Alkynyl sugar analogs for the labeling and visualization of glycoconjugates in cells, Proc Natl Acad Sci 104, 2614.

Before Starting

Materials Required but Not Provided

- Tissue culture treated, polystyrene culture dishes (recommended when use of minimum volume is required, because the hydrophilic surface allows for smaller volumes of media to cover the surface)
- DMSO (dimethyl sulfoxide)
- PBS (phosphate buffered saline, Cat. nos. 14190-144 or 14190-250)
- Fixative such as 4% paraformaldehyde in PBS
- Permeabilization agents such as Triton® X-100 or Saponin
- 3% bovine serum albumin (BSA) in PBS
- Methionine-free medium, if Click-iT® AHA or HPG is used. For example:
  - Dulbecco's Modified Eagle Medium (D-MEM contains 4,500 mg/L D-glucose, without L-glutamine, sodium pyruvate, L-methionine, and L-cysteine, Cat. no. 21013)
  - RPMI Medium 1640, contains no L-methionine (Cat. no. 050001DJ)

If proteins will be analyzed by standard protein biochemistry techniques such as gel electrophoresis and western blotting:

- 1% SDS in 50 mM Tris-HCl, pH 8.0
- Protease inhibitors (optional, but recommended)
- Phosphatase inhibitors (optional, but recommended)
- Probe sonicator capable of fitting into a 1.5 mL microcentrifuge tube to break up cellular DNA or Benzonase® endonuclease
- Methanol, chloroform, and 18 megaOhm water for protein precipitation
- SDS-PAGE gels, buffers, and protein standards (visit www.invitrogen.com for details)

Handling azides

When handling any azide-modified molecule, it is important to minimize exposure to light prior to the click reaction.

Experimental Protocols

Amount of Starting Material

For imaging, culture cells on cover slips or other desired imaging-compatible surface. For flow, approximately 1×10^6 cells are sufficient for one detection reaction using the Click-iT® Cell Reaction Buffer Kit (Cat. no. C10269).

For 1D or 2D gels, 1×10^6 cells are required to prepare 100 µg of lysate, which is sufficient for one detection reaction using the Click-iT® Protein Reaction Buffer Kit (Cat. no. C10276). You can find additional information for protein labeling and detection with click chemistry in the primary literature. Some references are provided in Table 4 on the previous page.

Metabolic Labeling of Cells

1.1 Solubilize the Click-iT® metabolic labeling reagent with DMSO to make a 500X or 1,000X stock solution. This process ensures that the DMSO concentration is not more than 0.1–0.2% in the cell culture. Aliquot and store any unused reagent at –20°C. When stored as directed, this stock solution is stable for up to 1 year.

1.2 If using Click-iT® AHA or HPG, wash cells once with warm PBS, add methionine-free medium to the cells and incubate the cells at 37°C for 30–60 minutes to deplete methionine reserves.

If not using Click-iT® AHA or HPG, proceed to step 1.3.

1.3 Add the desired concentration of metabolic labeling reagent to the cell medium, gently mix, and incubate at 37°C, 5% CO_2 for the desired time. Refer to Table 2 for example working concentrations, incubation times, and general conditions for using Click-iT® detection reagents or Table 4 for additional references.

1.4 For imaging or flow analysis, proceed to step 2.1. For 1D or 2D gel analysis, proceed to step 3.1 for suspension cells, or to step 4.1 for adherent cells.

Preparing samples for imaging or flow cytometry

2.1 Wash the cells with PBS.

2.2 Fix the cells with 4% paraformaldehyde in PBS for 15 minutes.

2.3 Permeabilize the cells with 0.25% Triton® X-100 in PBS or 1% BSA/0.1% saponin in PBS for 15 minutes.

Note: The click chemistry reagents are compatible with all other fixation and permeabilization protocols.

2.4 Wash the cells with 3% BSA in PBS.

2.5 The cells are now ready for the detection reaction with the corresponding azide- or alkyne-tagged detection molecule (Table 3) using the Click-iT® Cell Reaction Buffer Kit (Cat. no. C10269).

Preparing samples for 1D or 2D gel analysis

Harvesting Suspension Cells

3.1 Pellet the cells by centrifugation at 400 × *g* for 5 minutes. Discard the supernatant.

3.2 Resuspend the cell pellet in PBS by gently pipetting up and down using 5 mL PBS for cells from a 100 mm dish or 1 mL PBS per well for cells from a 6-well plate.

3.3 Pellet the cells by centrifugation at 400 × *g* for 5 minutes. Discard the supernatant.

3.4 Repeat the PBS wash 2 more times (steps 3.2–3.3) for a total of 3 washes to remove serum.

3.5 Pellet the cells by centrifugation at 400 × *g* for 5 minutes. Discard the supernatant. The cell pellet can be used directly in step 5.2 or flash frozen and stored at –80°C until use.

3.6 Proceed to step 5.1 to lyse the cells.

Harvesting Adherent Cells

Note: If analyzing cell surface proteins, do **not** use trypsin to detach cells, because trypsin cleaves cell surface proteins. You can lyse the cells directly in the culture dish, or, if desired, use a non-enzymatic dissociation buffer or a cell scraper, and pellet the cells. You can use the cell pellet directly in step 5.2, or flash freeze and store it at –80°C until use.

4.1 Remove the medium and wash the cells three times with PBS.

4.2 Proceed immediately to step 5.1 to lyse the cells for preparing samples for the Click-iT® detection reaction.

Lysing Cells

Do **not** use DTT, TCEP, or β-mercaptoethanol because they will reduce the azide.

5.1 Prepare the lysis buffer by adding protease and phosphatase inhibitors at appropriate concentrations to 1% SDS in 50 mM Tris-HCl, pH 8.0. You need 500 µL lysis buffer per 100 mm dish and 200 µL lysis buffer per well of a 6-well plate.

Note: Protease and phosphatase inhibitors are optional but recommended to ensure sample integrity.

If a probe sonicator is not available, add 250 U of Benzonase® endonuclease per mL of lysis buffer.

5.2 For adherent cells (from step 4.1), add 500 µL lysis buffer per 100 mm plate or 200 µL lysis buffer per well of a 6-well plate to the labeled cells. If adding the lysis buffer directly to the plate, tap or rotate the plates so the lysis buffer covers the bottom surface of the plate.

For suspension cell pellet (from step 3.5), add 50 µL lysis buffer per 1×10^6 cells.

5.3 Incubate the cells for 15–30 minutes on ice, then tilt the plates and pipet the lysate into a 1.5 mL microcentrifuge tube. If the lysis buffer does not contain Benzonase® endonuclease, the lysate may be very viscous due to the DNA from the lysed cells.

If using Benzonase® endonuclease, proceed to step 5.5.

5.4 Sonicate the lysate with a probe sonicator to solubilize the proteins and disperse the DNA.

5.5 Vortex the lysate for 5 minutes.

5.6 Centrifuge the cell lysate at 13,000–18,000 × *g* at 4°C for 5 minutes.

5.7 Transfer the supernatant to a clean tube and determine the protein concentration using the EZQ® Protein Quantitation Kit (Cat. no. R33200) or another method. The EZQ® Protein Quantitation Kit is compatible with 1% SDS present in the sample, and it requires only 1 µL of sample. Ideally, the protein concentration should be 1–2 mg/mL.

The protein sample is now ready for reaction with an azide or alkyne detection molecule (Table 3) using the Click-iT® Protein Reaction Buffer Kit (Cat. no. C10276).

If you are not immediately using the prepared protein sample for the Click-iT® detection reaction, store the sample at –20°C for up to 2 weeks. If you wish to store the sample for more than 2 weeks, proceed to step 6.1 to precipitate the sample.

Optional: Precipitating the Proteins

Precipitation step is not necessary if you plan to use the sample immediately in the Click-iT® detection reaction or if you are storing the sample at –20°C for up to 2 weeks.

6.1 Add 200 µL of the lysate from step 5.7 to a 1.5 mL microcentrifuge tube.

6.2 Add 600 µL of methanol to the tube, and vortex briefly.

6.3 Add 150 µL of chloroform to the tube, and vortex briefly.

6.4 Add 400 µL of 18 megaOhm water to the tube, and vortex briefly.

6.5 Centrifuge the tube for 5 minutes at 13,000–18,000 × *g*. Carefully remove and discard as much of the upper aqueous phase from the tube as possible, while leaving the interface layer containing the protein precipitate intact.

6.6 Add 450 µL of methanol to the tube and vortex briefly.

6.7 Centrifuge the tube for 5 minutes at 13,000–18,000 × *g* to pellet the protein. Discard the supernatant.

6.8 Cover the tube with a lint-free tissue and keep the tube cap open. Allow the pellet to air-dry, for 15 minutes to overnight.

6.9 Cap the tube and store the sample at –20°C until use.

Resolubilizing Precipitated Proteins

Perform this step if you have precipitated the proteins (steps 6.1–6.9).

7.1 On the day of the Click-iT® detection reaction, solubilize the proteins with 100 µL of 1% SDS in 50 mM Tris-HCl, pH 8.0 to obtain a 2–4 mg/mL protein solution.

7.2 Vortex the solution for 10–15 minutes. Centrifuge the solution for 5 minutes at 13,000–18,000 × *g* to remove any unsolubilized protein. If a significant pellet is visible, you may need to vortex for a longer time or sonicate to solubilize more protein. Repeat the centrifugation step after any additional solubilization efforts.

The supernatant is now ready for reaction with an azide or alkyne detection molecule (Table 3) using the Click-iT® Protein Reaction Buffer Kit (Cat. no. C10276).

References

1. ChemBioChem 4, 1147 (2003); **2.** J Am Chem Soc 125, 3192 (2003); **3.** Angew Chem Int Ed Engl 41, 2596 (2002); **4.** Angew Chem Int Ed Engl 40, 2004 (2001); **5.** J Am Chem Soc 130, 11576 (2008); **6.** Proc Natl Acad Sci 103, 9482 (2006).

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat. no.	Product Name	Unit Size
C10102	Click-iT® AHA (L-azidohomoalanine) *for nascent protein synthesis*	5 mg
C10186	Click-iT® HPG (L-homopropargylglycine) *for nascent protein synthesis*	5 mg
C10248	Click-iT® farnesyl alcohol, azide *mixed isomers*	1 mg
C10249	Click-iT® geranylgeranyl alcohol, azide *mixed isomers*	1 mg
C10264	Click-iT® fucose alkyne (tetraacetyl fucose alkyne)	5 mg
C10265	Click-iT® palmitic acid, azide (15-azidopentadecanoic acid)	1 mg
C10268	Click-iT® myristic acid, azide (12-azidododecanoic acid)	1 mg
C33365	Click-iT® GalNAz metabolic glycoprotein labeling reagent (tetraacetylated *N*-azidoacetylgalactosamine) *for *O*-linked glycoproteins* *5.2 mg*	1 each
C33366	Click-iT® ManNAz metabolic glycoprotein labeling reagent (tetraacetylated *N*-azidoacetyl-*D*-mannosamine) *for sialic acid glycoproteins* *5.2 mg*	1 each
C33367	Click-iT® GlcNAz metabolic glycoprotein labeling reagent (tetraacetylated *N*-azidoacetylglucosamine) *for *O*-GlcNAC-modified proteins* *5.2 mg*	1 each
Related Products		
A10266	Alexa Fluor® 488 azide (Alexa Fluor® 488 5-carboxamido-(6-azidohexanyl), bis(triethylammonium salt))	0.5 mg
A10267	Alexa Fluor® 488 alkyne (Alexa Fluor® 488 5-carboxamido-(propargyl), bis(triethylammonium salt))	0.5 mg
A20012	Alexa Fluor® 555 azide, triethylammonium salt	0.5 mg
A20013	Alexa Fluor® 555 alkyne, triethylammonium salt	0.5 mg
A10270	Alexa Fluor® 594 azide (Alexa Fluor® 594 carboxamido-(6-azidohexanyl) bis(triethylammonium salt))	0.5 mg
A10275	Alexa Fluor® 594 alkyne (Alexa Fluor® 594 carboxamido-(5-(and 6)-propargyl) bis(triethylammonium salt))	0.5 mg
A10277	Alexa Fluor® 647 azide, triethylammonium salt	0.5 mg
A10278	Alexa Fluor® 647 alkyne, triethylammonium salt	0.5 mg
B10184	biotin azide (PEG_4 carboxamide-6-azidohexanyl biotin)	1 mg
B10185	biotin alkyne (PEG_4 carboxamide-propargyl biotin)	1 mg
C10269	Click-iT® Cell Reaction Buffer Kit	1 kit
C10276	Click-iT® Protein Reaction Buffer Kit	1 kit
O10180	Oregon Green® 488 azide (Oregon Green® 488 6-carboxamido-(6-azidohexanyl), triethylammonium salt) *6-isomer*	0.5 mg
O10181	Oregon Green® 488 alkyne *6 isomer*	0.5 mg
T10182	tetramethylrhodamine (TAMRA) azide (tetramethylrhodamine 5-carboxamido-(6-azidohexanyl)) *5-isomer*	0.5 mg
T10183	tetramethylrhodamine (TAMRA) alkyne (5-carboxytetramethylrhodamine, propargylamide) *5-isomer*	0.5 mg
R33200	EZQ® Protein Quantitation Kit	1 kit
14190-144	Dulbecco's Phosphate Buffered Saline, 1X without Calcium Chloride without Magnesium Chloride	500 mL
14190-250	Dulbecco's Phosphate Buffered Saline, 1X without Calcium Chloride without Magnesium Chloride	10 × 500 mL

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:
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Technical Service:
8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

For country-specific contact information, visit www.invitrogen.com.

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