

Amine-Reactive Probes

Table 1. Contents and storage information.

Materials	Amount	Concentration	Storage Upon Receipt	Stability
Reactive dye (lyophilized solids)	Various, see product label	Not applicable	<ul style="list-style-type: none"> • ≤-20°C • Desiccate • Protect from light 	When stored as directed, dyes are stable for 6–12 months.
Spectral data: See Table 2 in the <i>Appendix</i> .				

Introduction

Molecular Probes manufactures a wide variety of amine-reactive fluorescent dyes, biotins, and other haptens for conjugation to proteins, amine-modified oligonucleotides, or other amine-containing compounds. This manual describes protocols for conjugating amine-reactive compounds to 10 mg of an IgG antibody or to 100 µg of an amine-modified oligonucleotide.

There are three major classes of commonly used reagents to label amines: active esters (which include succinimidyl esters (SE), sulfosuccinimidyl esters (SSE), tetrafluorophenyl esters (TFP), and sulfodichlorophenol esters (SDP)), isothiocyanates (ITC), and sulfonyl chlorides (SC). Of the three major classes, active esters are the preferred reagents for conjugates as they produce stable carboxamide bonds.¹ Of the four active esters, SDP esters are preferred as they are significantly less susceptible to hydrolysis than succinimidyl esters and TFP esters, thus providing greater control or more reaction time in aqueous-based reactions. Other amine-reactive groups include dichlorotriazines, aryl halides, and acyl azides. In addition, we have developed water-soluble amine-reactive forms of some of our popular hydrophobic dyes. These dyes have an amine-reactive 4-sulfo-2,3,5,6-tetrafluorophenyl (STP) ester group and may be a good alternative for labeling biomolecules in the absence of organic solvents. For more details and a complete list of our amine-reactive compounds, refer to *The Handbook, A Guide to Fluorescent Probes and Labeling Technologies*, available upon request or at our website at www.invitrogen.com. You can find further information on making bioconjugates in Hermanson, *Bioconjugate Techniques*, Academic Press, 1996 (Cat. no. B7884).

Labeling Proteins

Materials Required

Protein

You may conjugate amine-reactive reagents with virtually any protein or peptide. The following protocol is optimized for IgG antibodies. You may scale the reaction for any amount of protein, but the concentration of the protein should be at least 2 mg/mL for optimal results.

Reactive Dye

TFP, SDP, and succinimidyl esters are preferred for the conjugation to proteins because they form a very stable amide bond between the dye or hapten and the protein. Isothiocyanates are also commonly used, although the resulting thiourea product has been reported to deteriorate over time. Sulfonyl chlorides and acid halides are more reactive and may allow conjugation to aromatic amines. Sulfonyl chlorides form very stable sulfonamides that can survive complete protein hydrolysis, but since they are more difficult to work with, we do not recommend these for most routine conjugations with proteins.

Solvent

For the most part, reactive dyes and haptens are hydrophobic molecules and should be dissolved in high-quality, anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO). *Caution: Do not use DMSO with sulfonyl chlorides, as it reacts with them.*

Reaction Buffer

Amine-reactive reagents react with non-protonated aliphatic amine groups, including the amine terminus of proteins and the ϵ -amino group of lysines. The ϵ -amino group has a pK_a of around 10.5; in order to maintain this amine group in the non-protonated form, the conjugation must take place in a buffer with slightly basic pH. It is important to avoid buffers that contain primary amines, such as Tris, as these will compete for conjugation with the amine-reactive compound. We recommend the following buffers for conjugation with amine-reactive compounds with proteins:

- 0.1–0.2 M sodium bicarbonate buffer, pH 9.0 for TFP esters, isothiocyanates, sulfonyl chlorides and dichlorotriazines
- 0.1–0.2 M sodium bicarbonate buffer, pH 8.3 for succinimidyl, STP, and SDP esters

You may achieve more specific labeling of the amine terminus using a buffer closer to neutral pH, as the pK_a of the terminal amine is lower than that of the lysine ϵ -amino group.

Stop Reagent (Optional)

You may use 1.5 M hydroxylamine, pH 8.5, to terminate the reaction and to remove weakly bound probes. To prepare this reagent, dissolve hydroxylamine hydrochloride at 210 mg/mL in distilled water and adjust the pH to 8.5 with 5 M NaOH. Dilute the resulting 3 M solution with an equal volume of distilled water to get 1.5 M hydroxylamine, pH 8.5. Prepare this reagent freshly before use (see step 1.5 below).

Purification

You can easily separate a typical labeled antibody from free dye using a gel filtration column, such as Sephadex™ G-25, BioGel® P-30, or equivalent, equilibrated with the buffer of your choice. For much smaller or larger proteins, select a gel filtration media with a suitable molecular weight cutoff, or purify the conjugate by extensive dialysis (see step 2.1 below).

Labeling Protocol

You may scale this procedure up or down, maintaining the same molar ratios of reagents. It is important to consider that the number and surface position of the amines will vary greatly among proteins and even among different IgGs, as will the reactivity of the dyes. We recommend that you try three different degrees of labeling whenever possible, using three different molar ratios of the reactive reagent to protein, and base future protocols on the amount of reagent that gives the most satisfactory results for your specific protein. Reviews by Brinkley and by Haugland provide comprehensive surveys of procedures and reagents for protein conjugate preparation.^{2,3}

- 1.1 Dissolve ~10 mg of the protein in 1 mL of 0.1 M sodium bicarbonate buffer.** The protein concentration in the reaction should usually be 5–20 mg/mL. The kinetics and success of the reaction are highly concentration dependent. Concentrations lower than 2 mg/mL will greatly decrease the efficiency of the reaction.

Protein solutions must be free of any amine-containing substances such as Tris, glycine, ammonium ions, or stabilizing proteins such as bovine serum albumin. You can dialyze antibodies that have been previously dissolved in buffers containing amines against 10–20 mM phosphate-buffered saline (PBS), and you can obtain the desired pH for the reaction by adding 0.1 mL of 1 M sodium bicarbonate buffer (pH 8.3–9.0) for each mL of antibody solution. The presence of low concentrations of sodium azide (<3 mM) or thimerosal (<1 mM) will not interfere with the conjugation reaction.

- 1.2 Dissolve the amine-reactive compound in DMF or (except for sulfonyl chlorides) DMSO at 10 mg/mL.** For a typical reaction, dissolve 5 mg of dye in 0.5 mL of DMF or DMSO. Dissolve the dye immediately before starting the reaction as reactive compounds are not very stable in solution. You can prepare stock solutions of dyes with an STP ester reactive group in deionized water. Briefly sonicate or vortex.

- 1.3 While stirring or vortexing the protein solution (step 1.1), slowly add 50–100 µL of the reactive dye solution (step 1.2).** This volume corresponds to 0.5–1 mg of amine-reactive dye. In general, about one-fourth to one-third of the reactive dye will conjugate to the protein. This percentage may be higher with isothiocyanates. Variations due to the different reactivities of both the protein and the labeling reagent may occur, which may necessitate optimization of the dye-to-protein ratio used in the reaction.

- 1.4 Incubate the reaction for 1 hour at room temperature with continuous stirring.** For sulfonyl chlorides, incubate at 4°C, with continuous stirring.

- 1.5 *Optional:* Stop the reaction by adding 0.1 mL of freshly prepared 1.5 M hydroxylamine, pH 8.5, and incubate the hydroxylamine-containing reaction for one hour at room temperature.** For Rhodamine Green™ conjugates, incubate the hydroxylamine-containing reaction overnight at 4°C.

Note: Treatment with hydroxylamine at this stage is required to remove the trifluoroacetyl protecting groups of Rhodamine Green™ (Cat. no. R6112) succinimidyl ester. Hydroxylamine may also remove dye from unstable conjugates with tyrosine, serine, threonine and histidine.

Separating the Conjugate from Unreacted Labeling Reagent

We recommend using Sephadex™ G, BioGel® P, or equivalent gel filtration media with an appropriate molecular weight cutoff.

- 2.1** Equilibrate a 10 × 300 mm column with PBS or buffer of choice. The excluded fraction, which corresponds to the first fluorescent band to elute, is the conjugate. If you are conjugating a dilute antibody, you may want to purify the conjugate by extensive dialysis to avoid further dilution.

Note: You can obtain microdialysis apparatus for small volumes of proteins, *e.g.*, 10–500 µL, from Pierce Chemical Company (www.piercenet.com) and Spectrum Laboratories (www.spectrapor.com).

If you prefer to purify your conjugate by column chromatography, after elution, add bovine serum albumin (BSA) or any other stabilizer of choice to a final concentration of 1–10 mg/mL to prevent denaturation.

Storing the Protein Conjugate

Store the conjugates under the same conditions used for the parent protein. For storage in solution at 2–6°C, add sodium azide (2 mM final concentration) as a preservative. Since azide is an inhibitor of horseradish peroxidase (HRP), substitute thimerosal as a preservative for conjugates that are derived from HRP or those that will be used for experiments in which HRP is present. It may be necessary to remove the preservatives prior to use to avoid inhibitory effects in applications in which conjugates are added to live cell specimens.

Determining the Degree of Labeling

You may need to optimize the labeling efficiency to achieve the desired results in your application. You can determine the relative efficiency of a labeling reaction by measuring the absorbance of the protein at 280 nm and the absorbance of the dye at its excitation maximum (λ_{max}). Using the Beer-Lambert law, you can calculate the approximate number of dye molecules per protein molecule:

$$A = \epsilon \times \text{path length} \times \text{concentration}$$

where ϵ is the extinction coefficient in $\text{cm}^{-1}\text{M}^{-1}$. You also need to make a correction for the absorbance of the dye at 280 nm (see below). In the case of a biotinylated conjugate, you can measure the degree of labeling with HABA reagent as described in the protocol of our FluoReporter® Biotin-XX Protein Labeling Kit (Cat. no. F2610) and elsewhere.⁴

3.1 Measure the absorbance of the protein–dye conjugate at 280 nm (A_{280}) and at the λ_{max} for the dye (A_{max}). Dilute the protein–dye conjugate to approximately 0.1 mg/mL. Dilute only as much as you need to make the measurement. The λ_{max} values for commonly used fluorophores are given in the *Appendix*. Consult *The Handbook, A Guide to Fluorescent Probes and Labeling Technologies*, or our website at www.invitrogen.com for information about other dyes.

3.2 Determine the concentration of the protein in mg/mL.

a. Correct for the contribution of the dye to the absorbance at A_{280} .

$$A_{\text{protein}} = A_{280} - A_{\text{max}} (\text{CF})$$

$$\text{CF} = \frac{A_{280 \text{ free dye}}}{A_{\text{max free dye}}}$$

Note: CF values for commonly used fluorophores are listed in the *Appendix*.

b. Calculate the protein concentration assuming $1.4 A_{\text{protein}} \text{ units} = 1 \text{ mg/mL}$. This value is correct for IgG antibodies, but may be different for your protein.

3.3 Calculate the degree of labeling (D.O.L.):

$$\text{DOL} = \frac{A_{\text{max}} \times \text{MW}}{[\text{protein}] \times \epsilon_{\text{dye}}}$$

where MW = the molecular weight of the protein, ϵ_{dye} = the extinction coefficient of the dye at its absorbance maximum, and the protein concentration is in mg/mL.

Labeling Amine-Modified Oligonucleotides

Materials Required

Oligonucleotide

The oligonucleotide must be synthesized with an amine group on the 5' end. The following protocol has been optimized for use with 100 µg of oligonucleotide.

Reactive Dye

Succinimidyl esters are preferred for the conjugation to oligonucleotides because they are easy to use, and form a very stable amide bond between the dye or hapten and the amine-modified oligonucleotide. For DNA sequencing applications, the BODIPY® dyes are useful because they are isomerically pure and cause little perturbation to the mobility of DNA fragments.⁵ We also offer 5-FAM, 6-TAMRA, 6-ROX, and 6-JOE, the traditional fluorophores used in oligonucleotide labeling and automated DNA sequencing applications.⁶⁻⁹ The Alexa Fluor® series of dyes provides very bright, photostable fluorescence; however, because the fluorescence of the Alexa Fluor® 633 dye appears to be quenched by nucleic acids, we do **not** recommend this dye for labeling oligonucleotides.

Solvent

For the most part, reactive dyes and haptens are hydrophobic molecules and should be dissolved in high-quality, anhydrous dimethylsulfoxide (DMSO) before reaction with amine-modified oligonucleotides.

Reaction Buffer

Amine-reactive reagents react with the non-protonated amine group on the modified oligonucleotide. To maintain this amine group in the non-protonated form, the conjugation must take place in a buffer with slightly basic pH. For optimal results, we recommend using a tetraborate buffer at pH 8.5, rather than the bicarbonate buffers recommended for protein conjugations. Avoid buffers that contain primary amines, such as Tris, as these compete for conjugation with the amine-reactive compound.

Purifying the Amine-Modified Oligonucleotide

To ensure that the oligonucleotide is free of interfering compounds, especially amines, such as triethylamine or Tris, and ammonium salts, we strongly recommend extracting and precipitating the sample prior to initiating the labeling reaction. We suggest the following protocol for 0.1–1 mg oligonucleotide (3–30 A₂₆₀ units).

- 4.1 Dissolve the oligonucleotide in 100 µL of deionized water, and extract three times with an equal volume of chloroform.
- 4.2 Precipitate the oligonucleotide by adding one-tenth volume (10 µL) of 3 M NaCl and two and a half volumes (250 µL) of cold absolute ethanol. Mix well and incubate at ≤−20°C for 30 minutes.
- 4.3 Centrifuge the solution in a microcentrifuge at ~12,000 × g for 30 minutes.
- 4.4 Carefully remove the supernatant, rinse the pellet once or twice with cold 70% ethanol, and dry under vacuum.
- 4.5 Dissolve the dry pellet in deionized water to achieve a final concentration of 25 µg/µL (4.2 mM for an 18-mer). You may store this amine-modified oligonucleotide stock solution frozen at ≤−20°C.

Labeling Protocol

The protocol has been optimized for labeling 100 µg of an 5'-amine-modified oligonucleotide, 18 to 24 bases in length. You may label slightly shorter or longer oligonucleotides using the same procedure; however, adjustments to the protocol may be necessary for significantly shorter or longer oligonucleotides. You may scale the reaction up or down as long as you do not change the concentration of each component. The procedure has not been tested with oligonucleotides containing more than one amine. Following the labeling reaction, you may purify the conjugate from the reaction mixture using preparative gel electrophoresis or reverse-phase HPLC.

5.1 Prepare 0.1 M sodium tetraborate, pH 8.5 labeling buffer.

a. Make a 0.1 M sodium tetraborate buffer by dissolving 0.038 g of sodium tetraborate decahydrate for every mL of water.

b. Adjust pH with HCl to 8.5. Prepare this labeling buffer as close as possible to the time of labeling. Alternatively, you may divide the buffer into small aliquots, and freeze immediately for long-term storage.

Note: Exposure of this solution to air for a long time results in carbon dioxide absorption, which changes the pH of the buffer.

5.2 Dissolve 250 µg of the amine-reactive compound in 14 µL DMSO. For dinitrophenyl-X (DNP-X) succinimidyl ester, use 160 µg for 100 µg of oligonucleotide. For fluorescein, tetramethylrhodamine, Marina Blue®, FAM, or TAMRA succinimidyl ester, use 200 µg for 100 µg of oligonucleotide.

a. Allow amine-reactive compound to equilibrate to room temperature before opening the vial. Do **not** heat.

b. Dissolve the material by pipetting up and down, washing the sides of the vial. Texas Red® compounds tend to precipitate easily, and may require longer times to completely dissolve.

Note: It is important that you prepare the amine-reactive label freshly for each labeling reaction as reactive compounds are not stable in solution.

5.3 To the vial containing the reactive label in DMSO, add:

- 7 µL of deionized water
- 75 µL of labeling buffer (step 5.1)
- 4 µL of a 25 µg/µL oligonucleotide stock solution (step 4.5)

The reaction mixture may have a grainy appearance, but this does not adversely affect the conjugation. We strongly advise against attempting to improve the solubility of the label, because modifying the composition of the mixture can drastically reduce the labeling efficiency. You may scale the reaction up or down as long as the concentration of each component is not changed. Do not add more dye than recommended, as excess dye will not improve the labeling efficiency, and may make the purification more difficult.

5.4 Incubate the reaction for at least six hours (or overnight if more convenient) at room temperature.

a. Place the vial on a shaker oscillating at low speed, gently vortex to mix, or tap the vial every half hour for the first two hours to ensure that the reaction remains well mixed. Do not mix violently, as material may be left on the sides of the vial.

b. After six hours, 50–90% of the amine-modified oligonucleotide molecules will be labeled. Allowing the incubation to proceed overnight does not necessarily result in a greater labeling efficiency.

Ethanol Precipitating the Labeled Oligonucleotide

Following the reaction, the labeling mixture contains labeled oligonucleotide, unlabeled oligonucleotide, and unincorporated dye (or biotin or DNP). You can purify the labeled oligonucleotide from the reaction mixture using preparative gel electrophoresis or reverse-phase HPLC. Regardless of the purification method, we recommend ethanol precipitation as the first step.

- 6.1 Add one-tenth volume of 3 M NaCl and two and a half volumes of cold absolute ethanol to the reaction vial.
- 6.2 Mix well and incubate at $\leq -20^{\circ}\text{C}$ for 30 minutes.
- 6.3 Centrifuge the solution in a microcentrifuge at $\sim 12,000 \times g$ for a full 30 minutes. Loss of sample may occur if the centrifugation is not long enough.
- 6.4 Carefully remove the supernatant, rinse the pellet once or twice with cold 70% ethanol, and dry briefly. If the labeled oligonucleotide becomes completely dry, it will be difficult to redissolve.
 - Some unreacted labeling reagent may have precipitated over the course of the reaction or may be stuck on the walls of the reaction vial. **Completely** redissolve this material by extensive vortex mixing before centrifugation. Redissolving the labeling reagent ensures that the precipitated oligonucleotide will be minimally contaminated with unreacted label.
 - In some cases, the labeled oligonucleotide may have already precipitated onto the walls of the reaction tube. This precipitate will not dissolve with the addition of NaCl and ethanol—the precipitated product will remain on the walls of the tube; however, the free dye will dissolve and be eliminated. After centrifugation and rinsing, the pellet should be soluble.
 - Some reactions may benefit from a second ethanol precipitation to adequately eliminate the unreacted dye. In particular, when using tetramethylrhodamine (TAMRA), redissolve the oligonucleotide pellet in deionized water, and repeat the ethanol precipitation. This additional ethanol precipitation step is necessary because the tetramethylrhodamine labeling reagent has a tendency to adhere nonspecifically to the oligonucleotide. Similarly, a second ethanol precipitation is often appropriate when using the Texas Red[®]-X, BODIPY[®] 564/570, BODIPY[®] 581/591, or BODIPY[®] 630/650-X dyes.

Purifying the Labeled Oligonucleotide by HPLC

You can purify labeled oligonucleotides by reverse-phase HPLC using a standard analytical (4.6 × 250 mm) C8 column.

- 7.1 Dissolve the pellet from the ethanol precipitation (step 5.4) in 0.1 M TEAA (triethylammonium acetate).
- 7.2 Load the dissolved pellet onto the column in 0.1 M TEAA and run a linear 5–65% acetonitrile gradient over 30 minutes. This gradient is a 2% increase in acetonitrile per minute.
 - For oligonucleotides labeled with very hydrophobic dyes, like Texas Red[®] dye, you can achieve good separation running a faster gradient with up to a 3% increase per minute.
 - For separating oligonucleotides labeled with more hydrophilic dyes, like Marina Blue[®] dye, run a slower gradient, about 1% increase in acetonitrile per minute.
 - In all cases, the unlabeled oligonucleotide will migrate fastest, followed by the labeled oligonucleotide, and finally the free dye. For more details, refer to Oliver R.W.A., *HPLC of Macromolecules: A Practical Approach*, IRL Press (1989).

Purifying the Labeled Oligonucleotide by Gel Electrophoresis

- 8.1 To purify the labeled oligonucleotide by gel electrophoresis, pour a 0.5 mm–thick polyacrylamide slab gel. For oligonucleotides less than 25 bases in length, use 19% acrylamide, for oligonucleotides 25–40 bases, 15% acrylamide, and for oligonucleotides 40–100 bases, 12% acrylamide.
- 8.2 Resuspend the pellet from ethanol precipitation in 200 μ L of 50% formamide, and incubate at 55°C for 5 minutes to disrupt any secondary structure.
- 8.3 Load the warmed oligonucleotide onto the gel (you may need to use several wells) and load an adjacent well with 50% formamide plus 0.05% bromophenol blue.
- 8.4 Run the gel until the bromophenol blue indicator dye is two-thirds of the way down the gel. The bromophenol blue will migrate at approximately the same rate as the oligonucleotide.
- 8.5 Remove the gel from the glass plates, place on Saran Wrap, and lay it on a fluorescent TLC plate. Locate the labeled and unlabeled oligonucleotides by illuminating with a handheld UV source. Fluorophore-labeled oligonucleotides show fluorescence when illuminated with UV light.
- 8.6 Cut out the band containing the labeled oligonucleotide and purify by the “crush-and-soak” method or other suitable method. For more details, refer to Sambrook J., Fritsch E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory (1989).

References

1. *Bioconjug Chem* 6, 447 (1995); 2. *Bioconjug Chem* 3, 2 (1992); 3. *Methods Mol Biol* 45, 205 (1995); 4. *Methods Enzymol* 18A, 418 (1970); 5. *Science* 271, 1420 (1996); 6. *Anal Biochem* 238, 165 (1996); 7. *Anal Biochem* 223, 39 (1994); 8. *Nucleic Acids Res* 20, 2471 (1992); 9. *J Clin Microbiol* 37, 165 (1999).

Table 2. Spectral characteristics of common dyes. Values for other dyes may be found at our website (www.invitrogen.com).

Dye	λ_{max}^*	Em [*]	ϵ^\dagger	CF ₂₈₀ ‡
Alexa Fluor® 350	346	442	19,000	0.19
Alexa Fluor® 405	401	421	34,500	0.70
Alexa Fluor® 430	434	531	16,000	0.28
Alexa Fluor® 488	495	519	71,000	0.11
Alexa Fluor® 500	502	525	71,000	0.18
Alexa Fluor® 514	517	542	80,000	0.18
Alexa Fluor® 532	532	554	81,000	0.09
Alexa Fluor® 546	554	575	112,000	0.12
Alexa Fluor® 555	555	565	150,000	0.08
Alexa Fluor® 568	578	603	91,300	0.46
Alexa Fluor® 594	590	617	73,000	0.56
Alexa Fluor® 610-X	602	624	132,000	0.44
Alexa Fluor® 633	632	647	100,000	0.55
Alexa Fluor® 647	650	665	239,000	0.03
Alexa Fluor® 660	663	690	110,000	0.10
Alexa Fluor® 680	679	702	184,000	0.05
Alexa Fluor® 700	702	723	192,000	0.07
Alexa Fluor® 750	749	775	240,000	0.04
Alexa Fluor® 790	785	810	260,000	0.08
AMCA-X	346	442	19,000	0.19
BODIPY® 630/650	632	640	100,000	0.10
BODIPY® 650/665	651	660	100,000	0.13
BODIPY® FL	504	513	68,000	0.04
BODIPY® TMR	535	574	50,000	0.05
BODIPY® TR	588	617	45,000	0.22
Cascade Blue®	400	420	28,000	0.65
Dintrophenyl	349	NA	18,000	0.18
Fluorescein (FAM)	494	518	68,000 §	0.30
HEX	535	556	98,000	0.15
JOE **	520	584	75,000	0.25
Marina Blue®	365	460	20,000	0.30
Oregon Green® 488	496	524	70,000 §	0.12
Oregon Green® 514	511	530	70,000	0.19
Pacific Blue™	416	451	46,000	0.20
Pacific Orange™	400	551	24,500	0.60
Rhodamine Green™	505	527	68,000 §	0.19
QSY® 7	560	NA	90,000	0.22
QSY® 9	562	NA	85,000	0.23
QSY® 21	660	NA	89,000	0.32
QSY® 35	472	NA	23,500	0.19
ROX	575	602	82,000	0.16
Rhodamine Red™	570	590	120,000	0.17
TET	521	536	99,000	0.13
Tetramethylrhodamine (TAMRA)	555	580	65,000	0.30
Texas Red®	595	615	80,000	0.18

* Fluorescence excitation and emission maxima, in nm. † Extinction coefficient at ϵ_{max} in $\text{cm}^{-1}\text{M}^{-1}$.‡ Correction factor (A_{280} free dye / A_{max} free dye). § Measured at pH 8.0. ** Measured at pH 12.0. NA = Not applicable.

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