

[25] Synthesis of N-Substituted Glycine Peptoid Libraries

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Introduction

Oligomeric N-substituted glycines (NSG) or "peptoids" are a novel class of polymer that are ideally suited for the generation of diverse molecular libraries.¹ The compounds are stable,² easy to synthesize,³ and have potent biological activities.⁴ This chapter describes straightforward detailed protocols for synthesizing diverse peptoid libraries without any sophisticated equipment and with only a minimum of effort.

Background

In order for a class of molecules to be suitable for the generation of diverse libraries, ideally several criteria should be met. First, the compounds should be accessible by solid-phase synthesis to allow the resin-splitting methods⁵ of equimolar mixture synthesis to be used. Second, molecules with an oligomeric architecture allow a variety of chemical functionalities to be incorporated into a molecule with a *single linking chemistry*. This greatly simplifies the synthesis of libraries, especially with respect to automation.⁶ The chemistry used to couple the monomers must be high yielding and general enough to allow the inclusion of a wide variety of side-chain structures. Importantly, the monomers used must be readily available to facilitate the high-throughput synthesis of libraries; otherwise the most significant task in library production can easily become the preparation of

¹ R. J. Simon, R. S. Kania, R. N. Zuckermann, V. D. Huebner, D. A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg, C. K. Marlowe, D. C. Spellmeyer, R. Tan, A. D. Frankel, D. V. Santi, F. E. Cohen, and P. A. Bartlett, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9367 (1992).

² S. M. Miller, R. J. Simon, S. Ng, R. N. Zuckermann, J. M. Kerr, and W. H. Moos, *Bioorg. Med. Chem. Lett.* **4**, 2657 (1994).

³ R. N. Zuckermann, J. M. Kerr, S. B. H. Kent, and W. H. Moos, *J. Am. Chem. Soc.* **114**, 10646 (1992).

⁴ R. N. Zuckerman, E. J. Martin, D. C. Spellmeyer, G. B. Stauber, K. R. Shoemaker, J. M. Kerr, G. M. Figliozzi, D. A. Goff, M. A. Siani, R. J. Simon, S. C. Banville, E. G. Brown, L. Wang, L. S. Richter, and W. H. Moos, *J. Med. Chem.* **37**, 2678 (1994).

⁵ Á. Furka, M. Sebestyén, M. Asgedom, and G. Dibó, *Int. J. Pept. Protein Res.* **37**, 487 (1991).

⁶ R. N. Zuckermann, J. M. Kerr, M. A. Siani, and S. C. Banville, *Int. J. Pept. Protein Res.* **40**, 497 (1992).

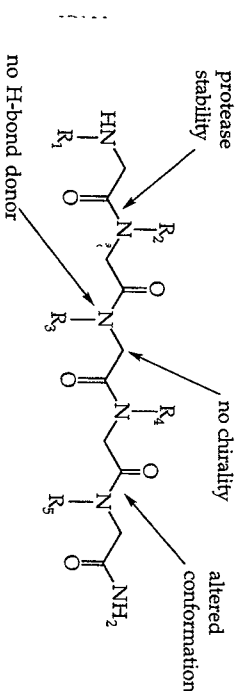


Fig. 1. Structure of an N-substituted glycine peptidic pentamer (C-terminal amide) and some major differences as compared to a peptide.

large monomer stocks by a team of synthetic chemists. Finally, the molecules generated should be hydrolytically and enzymatically stable.

Our efforts have focused on the synthesis of NSG peptidic oligomers because they fit the criteria just listed. These oligomers are structurally similar to peptides, but have several major differences, as outlined in Fig. 1. The original method¹ for the synthesis of oligomeric NSG peptides was analogous to standard solid-phase methods for peptide synthesis. Specifically, *N*^α-9-fluorenylmethoxy-carbonyl (Fmoc)-protected monomers were coupled to the secondary amino group of the resin-bound peptidic chain, followed by removal of the Fmoc group. A disadvantage of this approach, however, was the necessity of preparing suitable quantities of diverse sets of protected NSG building blocks.

In a recent synthetic advance,³ however, it was found that each NSG monomer could be assembled from two readily available "submonomers" in the course of extending the NSG polymer (Fig. 2). Each cycle of monomer addition consists of two steps: acylation and nucleophilic displacement. This is unlike peptide synthesis in that no backbone-protecting groups are used. Thus, oligomeric NSGs can be considered as alternating condensation copolymers of a haloacetic acid and a primary amine. The α -haloacetyl submonomer is common to all cycles of chain extension. Each R-NH₂ submonomer is simple in structure and thousands are commercially available, providing a readily available source of diversity for peptidic oligomers. Thus, NSG oligomer synthesis has been dramatically simplified.

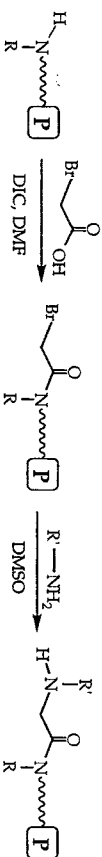


Fig. 2. Two-step cycle for the synthesis of an N-substituted glycine on a solid support.

Synthesis of Individual Peptides

The synthesis of individual peptidic oligomers is performed with simple equipment and apparatus. Although the chemistry is different, the mechanics of the synthesis are similar enough to peptide synthesis that the procedures described below can be adapted to most commercial peptide synthesizers. A few programming changes are necessary to allow putting amine solutions in the amino acid bottles and bromoacetic acid and *N,N'*-diisopropylcarbodiimide (DIC) in the deprotection reagent and activator bottles, respectively. The automated synthesis of peptides will not be described further here. This chapter emphasizes protocols that require little in the way of resources to get started.

Fmoc-Rink amide resin (Novabiochem, San Diego, CA) is used as the solid support. This is the same resin that is used for the Fmoc synthesis of peptide C-terminal amides. The peptidic synthesis begins with the deprotection of the Fmoc group on the resin with 20% (v/v) piperidine/dimethylformamide (DMF). The amino resin is then acylated with bromoacetic acid. This is followed by nucleophilic displacement of the bromide with a primary amine to build the NSG monomer. The latter two steps are then continued in an iterative fashion to elaborate the desired oligomer. We have had success with many primary amines using this simple coupling scheme. Only a few classes of amines require protection of side-chain functionality. These include aliphatic hydroxyl groups, carboxylic acids, thiols, amines, and some heterocycles.

When making a large library with great diversity that incorporates many amines it is impractical to analyze the final mixture of products. In this regard it is essential to verify that any submonomer incorporated into a library design actually works in the synthesis of an individual NSG oligomer. We developed a scheme for testing amines by incorporating them in the intermediate position(s) of an individual NSG oligomer flanked by a well-behaved submonomer. For example, a common test sequence that we use is *N*-benzylglycine-test-*N*-benzylglycine-test-*N*'-benzylglycine. In this format the desired amine must both displace the bromide and be acylated two times within the sequence. The product pentamer will have a convenient retention time on reversed-phase HPLC due to the three benzyl groups, regardless of the hydrophobicity of the test amine. In addition, amines that fail in this system will usually give the *N*-benzylglycine trimer, an easily characterized product. The products of these test cases are typically characterized by analytical reversed-phase C₁₈ HPLC and electrospray mass spectrometry. Criteria for acceptance is confirmation of the expected molecular weight (electrospray MS), purity of at least 70% (HPLC), and a minimum yield (mass recovery) of 50%.

Most simple amines are very well behaved, leading to a single product in high yield in the previously mentioned test.³ These include the numerous straight and branched aliphatic amines, aromatic amines, amines containing protected aliphatic hydroxyls, protected amines, protected acids, and even volatile amines. A few representative amines from each class are shown in Table I. The protecting group on any submonomer should ideally be trifluoroacetic acid (TFA) labile under cleavage conditions. It is, of course, possible to deprotect further after cleavage from the resin. A number of amines such as mono-*tert*-butoxycarbonyl (Boc)-protected alkyldiamines (e.g., Boc-diaminopropane) (Fluka, Ronkonkoma, NY), as well as many protected acid side-chain amines (e.g., glycine-*tert*-butyl ester, β -alanine-*tert*-butyl ester) (Bachem Biosciences, King of Prussia, PA) are commercially available and deprotect under the cleavage conditions. Primary hydroxyl groups can be protected in one step (in the presence of a free amino group) with silyl ethers.⁴ Testing of individual oligomers has proven these types of protecting groups to work well in NSGs. Moreover, protection of most primary amines is generally a straightforward procedure and easily accomplished. Therefore, monomer diversity should not necessarily be limited to choosing only commercially available protected amines.

While many of these submonomer reactions are quite facile, the multiple parallel synthesis procedure used in the synthesis of a library requires conditions for which *every* reaction will go to completion. Our strategy, therefore, is to use high reagent concentrations and long reaction times to ensure that even the less nucleophilic amines react. In this regard we typically perform the acylation step twice per cycle and use the amine as a 1–2 *M* solution and a reaction time of 2 hr in the displacement step.

TABLE I
EXAMPLES OF DIVERSE AMINES

Amine	Compound class	Supplier
Aniline	Aromatic	Aldrich
Butylamine	Aliphatic, linear	Aldrich
Cyclopentylamine	Aliphatic, β -branched	Aldrich
1,3-Diaminopropane (Boc)	Charged, positive	Fluka
Ethanolamine (<i>O</i> -trisisopropylsilyl)	Aliphatic hydroxylic	None ⁴
Furfurylamine	Heterocyclic	Aldrich
Glycine (<i>O</i> -tBu)	Charged, negative	Bachem
Methylamine	Small, volatile	Aldrich
2,2-Diphenylethylamine	Bulky	Aldrich
Tyramine	Aromatic hydroxylic	Aldrich

Our first choice of solvent for the displacement step is dimethyl sulfoxide (DMSO) since most amines are soluble at the desired concentration. However, we have found that a few amines are not soluble in DMSO at this concentration and that a lower concentration or other solvent systems can be used. The lowest concentration should not be less than 0.5 *M*. Sonication of the amine/solvent in a warm water bath is an excellent aid in solubilizing the amines.

The testing of submonomers in individual oligomers has led to the discovery of two major competing side reactions, both a result of intramolecular cyclization reactions. The synthesis of dimeric NSGs often leads to formation of *N*-substituted diketopiperazines rather than the linear dimer. Submonomers whose side chain bear a nucleophile three or four atoms from the amino nitrogen are also prone to cyclizations to the main-chain bromoacetyl group. The submonomers *N*-*Im*-tritylhistamine, 2-(amino-methyl)benzimidazole, 2-(aminomethyl)pyridine, and 4-(2-aminoethyl)morpholine fall into this category. These examples illustrate the importance of testing all submonomers prior to incorporation in a library.

Peptoid Library Synthesis

The synthesis of an *N*-substituted glycine peptoid library is similar to the synthesis of individual compounds described earlier. The primary differences are that (1) many samples are handled in parallel, and (2) a resin-mixing/splitting step (Fig. 3) must be added (see procedure below).

In addition to these procedural changes, several important design criteria must be considered prior to the library synthesis. The first consideration is the size of the library, including the length of the oligomer and the number of amine submonomers. We have found it most convenient to work with 20–30 amine submonomers. In the resin-splitting scheme (Fig. 3) we usually do not combine the resins after the last monomer addition step. Thus, a library consisting of all possible trimers from 24 monomers ($24^3 = 13,824$ compounds) for general screening could be made as 24 pools of 576 compounds/pool in only three monomer addition cycles. The scale of the library synthesis (μmol of resin per synthesis) depends on the amount of material needed for screening. For example, we generally perform a trimer library synthesis on 2.4 mmol of resin, typically yielding about 1 ml of each of 24 pools, each at a concentration of 100 μM per compound. This quantity is sufficient for hundreds of screens when screened at 1 μM per compound. We have found that it is optimal to keep the number of compounds per pool to less than a thousand. This is not only to maintain the accuracy of biological assay data, but also for solubility reasons. If such a pool is found

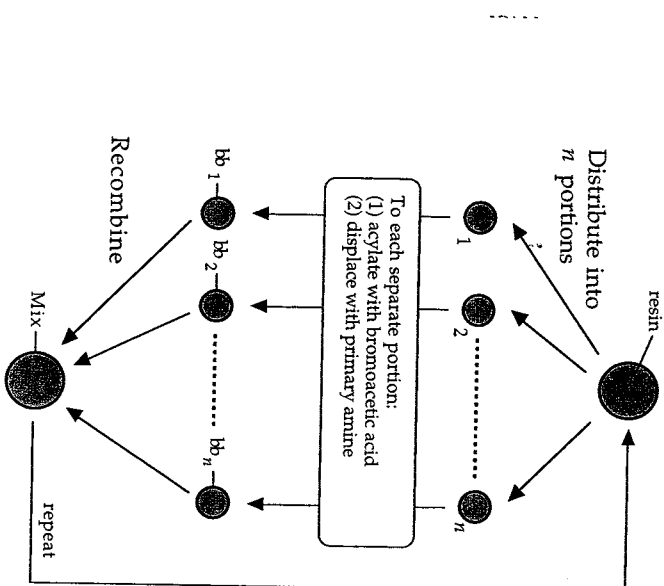


Fig. 3. Resin-splitting scheme for the synthesis of equimolar mixtures. For convenience, the acylation (step 1) can be performed prior to the distribution.

to be active, then it must be HPLC fractionated, affinity selected,^{7,8} or deconvoluted⁴ to find the active compound(s).

The nature of the amines chosen depends on the end use of the library. There are over 1000 amines commercially available. If nothing is known about the target protein then a set of amines as diverse as possible⁹ can be selected. If there are known pharmacophores, then some of these features can be incorporated.

General Experimental Procedures

Equipment for Peptoid Synthesis

10-ml fritted columns with Luer fitting (Kontes (Vineland, NJ) Econo-column or Burdick & Jackson (Muskegon, MI) solid-phase extrac-

⁷ R. N. Zuckermann, J. M. Kerr, M. A. Siani, S. C. Banville, and D. V. Santi, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 463 (1992).

⁸ J. M. Kerr, S. C. Banville, and R. N. Zuckermann, *Bioorg. Med. Chem. Lett.* **3**, 463 (1993).

⁹ E. J. Martin, J. M. Blaney, M. A. Siani, D. A. Spellmeyer, A. K. Wong, and W. H. Moos, *J. Med. Chem.* **38**, 1431 (1995).

tion vessel) fitted with a polypropylene stopcock to be used as a reaction vessel for up to 500 mg of resin
 125- to 150-ml fritted glass reaction vessel calibrated to hold 102 ml, for use as the "mixer"
 Caps or stoppers to seal tops of reaction vessels
 Test tube rack to hold reaction vessels, mounted onto an orbital shaker to simultaneously agitate 20–30 reaction vessels
 Argon gas supply
 Solvent dispensers (1–5 ml) for DMSO, DMF, and dichloromethane
 Adjustable pipettors to add reagents and to transfer resin
 Vacuum manifold with Luer fittings to drain reaction vessels
 Glass scintillation vials for cleavage reactions (must *not* have foil-lined caps)
 50-ml polypropylene centrifuge tubes for lyophilization
 5-ml polypropylene cryovials for final lyophilization and sample storage
 Magnetic stir bars ($\frac{1}{2}$ inch)
 Magnetic stirring plates
 Lyophilizer

Reagents

20% (v/v) piperidine in DMF for Fmoc group removal
 0.6 M bromoacetic acid in DMF (8.3 g bromoacetic acid in 100 ml DMF)
 3.2 M *N,N'*-diisopropylcarbodiimide (DIC) in DMF [1:1 (v/v) DIC/DMF]
 11 M KOH in water for neutralizing amine hydrochloride salts
 1–2 M amine solutions in DMSO
 95% trifluoroacetic acid (TFA)/5% (v/v) water for cleavage from resin
 65% 1,2-dichloroethane/35% DMF (v/v) "transfer solvent" for resin splitting and recombination

Resin Handling

All reactions and washings are performed at room temperature unless otherwise noted. Washing of the resin refers to the addition of a wash solvent (usually DMF or DMSO) to the resin, agitating the resin so that a uniform slurry is obtained (typically for about 20 sec), followed by thorough draining of the solvent from the resin. Solvents are best removed by vacuum filtration through the fritted bottom of the reaction vessel until the resin appears dry (typically about 5 sec). In all of our syntheses, resin slurries were agitated via bubbling argon up through the bottom of the fritted vessel. Since it may not be practical to construct or purchase a similar gas delivery manifold for all applications, we suggest that reaction vessels be placed in a rack on an orbital shaker whenever prolonged mixing is necessary. This will achieve effective mixing of the resin slurries. The reaction

vessels may be charged with argon gas and capped before leaving them to mix for an acylation or displacement cycle. Solvents used to dissolve reagents should be degassed prior to use by sonication under house vacuum for 5 min. For wash solvents, it is very convenient to have dispensers containing DMF, DMSO, and dichloromethane available with adjustable volumes (1–5 ml). Reaction vessels can be simultaneously drained on a vacuum manifold.

Special Amines

Methylamine is supplied as a 40% solution in water; this material is used neat from the bottle. Amine hydrochloride salts must be neutralized prior to use (see below).

Hydrochloride Salts

Dissolve or suspend each amine hydrochloride into DMSO to give a final concentration of 1–2 M. Add 0.95 M equivalents of an aqueous potassium hydroxide (KOH) solution (11 M) and vortex (potassium chloride will precipitate out as a white solid). Please note that reagent grade KOH typically contains 10–15% water. Spin the tubes in a benchtop centrifuge (~500 rpm, 2 min) and decant to separate the salt from the amine solution. Absolute dryness of the supernatants is not critical. A small volume of solution is typically lost through this process, so a little extra of these solutions is typically prepared.

Pausing a Synthesis

If a synthesis is halted and the resin is to be stored for a length of time (overnight), it is recommended that the resin be rinsed well with dichloromethane before storage. Resins containing residual DMF tend to form into a hard pellet. Resins that are to be stored may be further dried under high vacuum. It is advisable not to interrupt a synthesis at the dimer stage because dimers can cyclize on storage over a long period of time to form diketopiperazines.

Peptid Oligomer Synthesis

A fritted reaction vessel is charged with 100 mg (50 μ mol) of Fmoc-Rink amide resin (substitution level \approx 0.50 mmol/g resin). Two milliliters of DMF is added to the resin and this solution is agitated for 1–2 min to swell the resin. A glass rod may be used to break up chunks of resin, if necessary. The DMF is then drained. The Fmoc group is then removed by adding 2 ml of 20% piperidine in DMF to the resin. This is agitated for

1 min and then drained. Another 2 ml of 20% piperidine in DMF is added to the resin and agitated for 15 min and then drained. The resin is then washed with DMF (6 \times 2 ml).

The deblocked amine is then acylated by adding 850 μ l of 0.6 M bromoacetic acid in DMF to the resin followed by 200 μ l of 3.2 M DIC in DMF. This solution is agitated for 30 min at room temperature and then drained. This step is repeated a second time. The resin is then washed with DMF (2 \times 2 ml) and DMSO (1 \times 2 ml).

The acylation step is then followed by nucleophilic displacement with the first (C-terminal) amine. One milliliter of a 1–2 M solution of the desired amine in DMSO is added to the washed resin. This solution is agitated for 2 hr at room temperature and then drained. The resin is then washed with DMSO (2 \times 2 ml) and DMF (1 \times 2 ml). This completes one reaction cycle (Table II).

The second cycle is initiated by the acylation step with bromoacetic acid and DIC, followed by displacement with the second amine. This acylation/displacement cycle is repeated until the desired oligomer is obtained. After the final displacement reaction and resin washing, the resin is washed with dichloromethane (2 \times 2 ml). At this point the resin can be cleaved or it can be dried *in vacuo* for 2 hr. The resin can then be safely stored for several days at room temperature or in a refrigerator at this stage.

Cleavage for 50 μ mol Resin

The dried resin is placed in a glass scintillation vial containing a Teflon-coated micro stir bar, and approximately 5 ml of 95% TFA in water is

TABLE II
REACTION CYCLE FOR PEPTOID SYNTHESIS^a

Step	Reagent	Reaction time	Volume (ml)	Repetition
1	Deprotection 20% piperidine/DMF	1 min	2	1 \times
2	Wash DMF	15 min	2	1 \times
3	Bromoacetic acid 0.6 M bromoacetic addition acid/DMF	20 sec	2	6 \times
4	Activation 3.2 M DIC/DMF	—	0.20	—
5	Acylation —	30 min	—	2 \times
6	Wash DMF	20 sec	2	2 \times
7	Displacement DMSO	20 sec	2	1 \times
8	Wash 1–2 M amine/DMSO	2 h	1	1 \times
	DMF	20 sec	2	2 \times
	DMF	20 sec	2	1 \times

^a Volumes are for a 50- μ mol scale. Repeat steps 3–8 to elongate oligomer

added. (*Caution:* TFA is a strong corrosive and emits hazardous vapors. It must only be worked with in a well-ventilated fume hood.) Place the scintillation vials onto a stirring plate located in a fume hood. One stirring plate can accommodate four or five vials at a time. This solution is stirred for 20 min. Filter the cleavage mixture for each sample through an 8-ml solid-phase extraction (SPE) column fitted with a 20- μ m polyethylene frit into a 50-ml polypropylene conical centrifuge tube. The cleavage time may need to be lengthened, depending on which protecting groups are present in a particular library. The resin is then washed with 1 ml of the 95% TFA and the filtrates are combined. The filtrate is then diluted with an equal volume of water in the centrifuge tube. This solution is then frozen and lyophilized to dryness. By puncturing small holes in the caps of the vials we are able to directly lyophilize from the polypropylene tubes. The dried product is then dissolved in 10 ml of glacial acetic acid or 1:1 (v/v) water/ acetonitrile (brief sonication is a useful aid here) and again lyophilized to dryness. The twice dried product is then dissolved in 3 ml of glacial acetic acid or 1:1 (v/v) water/acetonitrile and transferred to a tared 5-ml cryovial (the kind with a silicone O ring are ideal) and then lyophilized to dryness, generally producing a white fluffy powder. The mass recovery can then be calculated and the product can remain in the cryovial for cold storage. For the purpose of calculating yields and molecular weights, it may be assumed that the product is the trifluoroacetate salt. We have found it very convenient to enter this data directly into a spreadsheet on a computer. Prior to the last lyophilization, HPLC and mass spectrometry (MS) samples should be prepared.

If the material is going to be used for testing in a biological assay, then DMSO is added to make a concentrated stock solution. Our goal for stock solutions is a concentration of 100 μ M per compound or greater. This 100 \times DMSO stock may be diluted 1:100 into buffer, yielding an assay solution that contains 1% DMSO and sample molecules at a concentration of 1 μ M per compound (a typical screening concentration). DMSO is a good choice for this because it dissolves peptoids very well and, when diluted, is compatible with most biological assays (at concentrations \leq 1%).

Oligomer Characterization

Individual peptoid oligomers are analyzed by reversed-phase HPLC on C_{18} columns (Vydac (The Separations Group, Hesperia, CA), 5 μ m, 300 \AA , 4.6×250 mm). A linear gradient of 0–80% B in 40 min is used at a flow rate of 1 ml/min (solvent A, 0.1% (v/v) TFA in water; solvent B, 0.1% (v/v) TFA in acetonitrile). Major peaks are collected and submitted to electrospray MS analysis to determine the molecular weights.

Resin-Splitting Procedure

The number of amine submonomers used at each position of a library (n) determines the number of vessels to split the resin into for each cycle. Resin splitting should be performed directly prior to the displacement step. Resin recombination should be performed directly after the displacement step. In other words, the acylation step can be performed in a single large reaction vessel or "mixer."

Resins are transferred by the isopycnic slurry method.⁶ A precise volume of resin slurry in a large reaction vessel is split into equal portions by volume. Thus, a fritted vessel or "mixer" with a capacity of 125 ml is calibrated to hold 102 ml. The vessel with resin is filled with the transfer solvent to the calibration mark, and the slurry is aliquoted. The amount of transfer solvent used depends on the amount of resin. Typically, 100 ml of solvent is used for every 3–4 g of resin.

Ensure that the resin has been rinsed and drained of solvent. Add "transfer solvent" to the 102-ml mark. Agitate the mixture and stir if any chunks of resin need to be broken up. A uniform isopycnic slurry of resin should be obtained. Line up n reaction vessels on a vacuum manifold (where n is the number of amines). Using a Pipetman, remove (100/ n) ml of the slurry and transfer it into a reaction vessel. Agitate the slurry in the mixer and remove a second aliquot of slurry, transferring it into a second reaction vessel. Repeat this process until all of the aliquots of slurry have been distributed. It is essential that the slurry in the large reaction vessel is agitated immediately before aliquots are removed. This will ensure that equal volumes of slurry contain equal mmols of resin, as the resin does settle slowly to the bottom of the vessel. There should be about 2 ml of slurry remaining at the bottom of the mixer. Drain the solvent from the mixer and repeat the procedure to ensure complete transfer of the resin. The reaction vessels should be labeled at this point to indicate which amine will be used for the displacement reaction in that vessel.

Resin Recombination

Add transfer solvent to a reaction vessel, agitate the slurry, and immediately transfer the entire volume into the mixer. Repeat this procedure to ensure complete transfer of the resin. Repeat for each of the reaction vessels.

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