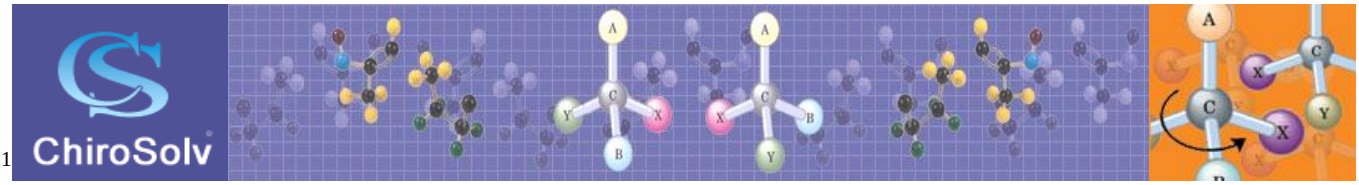

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ChiroSolv® Screen Kits Instruction Manual

Types of kits

ChiroSolv® kits are based on simple acid-base neutralization followed by re-crystallization in suitable solvent. The goal is to determine the optimum combination of resolving agent and solvent that allows quick crystallization of the chirally pure compound and to determine conditions permitting maximal recovery of the pure enantiomer. Acid kits are used with basic racemates, while base kits are used with the acidic racemates. Kits can also be used with neutral racemates of type alcohols, amino acids, aldehydes and ketones after doing some pre-processing" (described later).

Acid kits: Kits include a group of chirally pure acids and solvents combinations. They are used to resolve racemic bases. Each kit includes 8 different acids and 12 different solvents. Most of the acids used in these kits are easily available in bulk quantities and are commonly used in manufacturing processes.

Base kits: Kits include 4 plates of chirally pure bases (or amines). They are used to resolve racemic acids. Each kit includes 8 different amines and 12 different solvents. Most of the bases used in these kits are easily available in bulk quantities and are commonly used in manufacturing processes.

Strong acid kits: Kits include a group of chirally pure acids (with <3 pHs). They are used to resolve racemic bases. Each kit includes 8 different acids and 12 different solvents. Most of the acids used in these kits are easily available in bulk quantities and are commonly used in manufacturing processes.

Kits for solid racemate: Since it is very difficult to dispense a small quantity of racemate into each vial, most scientists prefer to dissolve the racemate into a solvent and then dispense the resultant solution into each vial. To allow scientists to dispense the racemate in this fashion, but to avoid having two different solvents in the vials (the solvent in which racemate is dissolved in; and the experimental solvent provided with the kit); ChiroSolve has designed kits for solid racemate where the resolving agents and solvents are provided in separate plates. These kits have two components:

1. Disposable 96 vial high-throughput format plates with resolving agents added to each vial. The vials are sealed with peelable and pierceable cap-mat/seal so that racemate dissolved in its solvent can be added easily.
2. Disposable 96 well plate which contains the needed solvent (200 µl quantity) in each well. The plate is sealed with peelable or pierceable cap-mat or seal, so that the solvents can be easily transferred into the above kit after the original solvent of the racemate solution has been evaporated.

Initially the 3 mmol of solid racemate is dissolved in the minimum amount of solvent that is most volatile and in which the racemate dissolves completely. This solution is then dispensed into 96 vials of the kit that contains resolving agents. Once this solvent is completely evaporated from the racemate solution, the solvents provided in the separate plate are dispensed in to the kit and the experiment is continued as described below. Note that the 96 well solvent plate has the exact same assay format and solvent in specific position within the plate must be dispensed into the vial in same position of the kit (e.g. solvent in A1 position must be dispensed into the vial marked as A1).

How to use kits

Each experiment needs about 0.03 mmol of unknown racemate in each of the 96 vials. It can be used directly with racemates of type acids and bases; while some preprocessing will be required for alcohols, amino acids, aldehydes and ketones. After following the steps described below, typically, only one or two vials will show maximum optical purity. One

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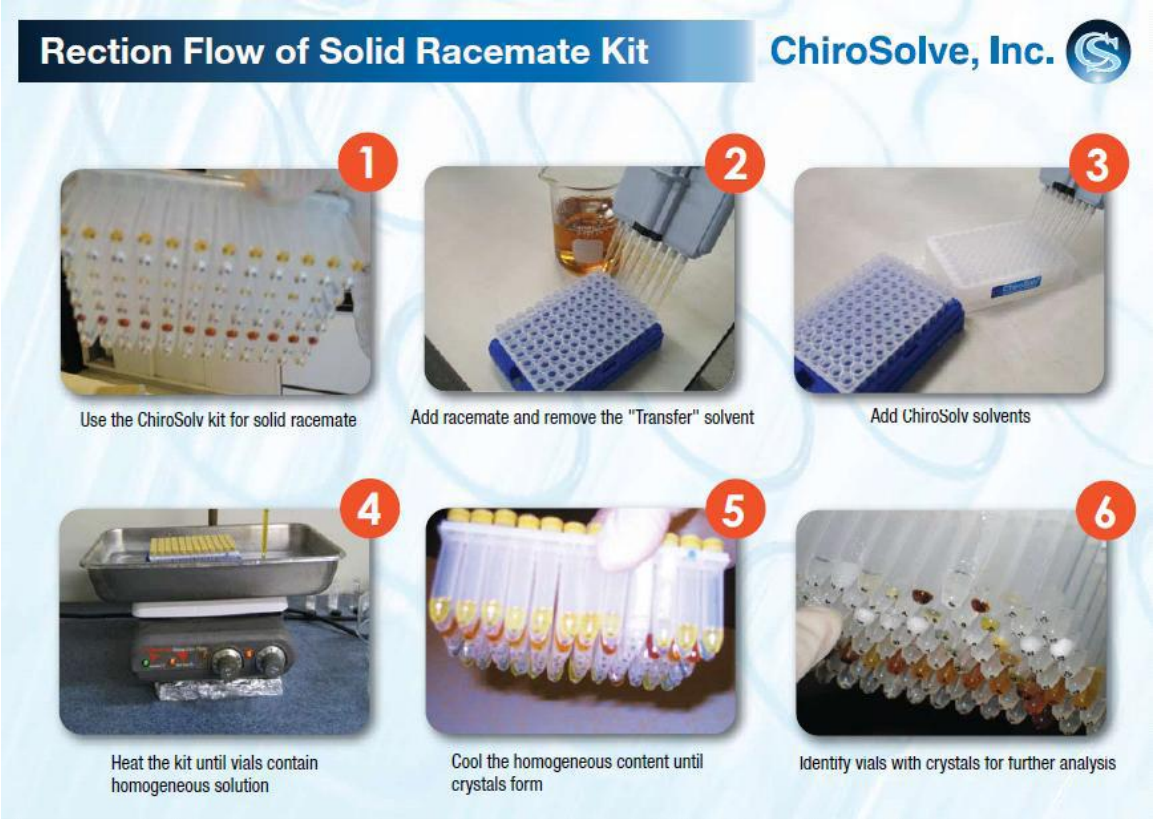
would be dextro (+) and other laevo (-) rotatory. Only the vials with desired enantiomer will have to be investigated further along with its' mother liquor for scale-up condition optimization. The experiment involves following steps:

1. Choose the right set of kits (A1, A2, A3, A4 or B1, B2, B3, B4) depending on whether the unknown racemate is base or acid respectively.
2. If the racemate is of type alcohol, amino acid, aldehyde or ketone, do the pre-processing as described below.
3. Add 0.03 mmol of your racemate to each of the 96 vials.
   - If the racemate is liquid, and you are using the kit for liquid racemate, simply dispense 0.03 mmol of the liquid in to each of the 96 vial by piercing through the seal/cap mat.
   - If the racemate is solid, and you are using the kit for solid racemate, dissolve the needed amount of racemate into a most volatile solvent that it can dissolve in using the minimum amount of solvent necessary. Remove the cap-mat or seal from the kit. Add the right amount of racemate solution so that when the solvent evaporates; there will be 0.03 mmol of the racemate in each of the tubes. Evaporate the solvent that the racemate was dissolved in completely. Now take the solvent plate; remove its seal; or pierce through each column using 8 channel pipetter (each column has same set of solvents in exact same order; so there won't be any contamination) and dispense into the vials of the kit in the exact same position vial.
4. Heat the rack along with its vials to 80º C (the optimum temperature for most of these experiments) or until the mixture becomes homogeneous.
5. Allow the kit to cool to ambient temperature. Then, if required, further cool it to 4º C and finally to 0º C and observe any crystallization. Vials with crystals are considered positive tests and need further investigation.
6. Using crystal initiation techniques, encourage more crystal formation. Vials with no crystals even after this effort are considered negative tests.
7. Select the vials with crystals (positive tests) note down the kit's bar-code along with the vial identification (located at the bottom of the vial).
8. For the selected vials in 7; separate out the crystals from the filtrate
9. If chiral HPLC, IR or CE are available, use the filtrate; or the crystals to do further analysis
10. If instruments in 9 above are not available, analyze each of the crystals separately after liberating enantiomers from it's' diastereomeric salts for specific rotation using polarimeter
11.



Pre-processing of Alcohols

Alcohol is neutral in functionality and it is usually resolved by conversion into the monoester of succinic or phthalic acid. This hydrogen succinate or phthalate then gets converted into diastereomeric salt with optically active bases.

Steps:

1. Treat the racemic alcohol with 1X1 molar ratio of anhydride and greater than 1X1 molar ratio of pyridine. You can also use 1X1 molar ratio of succinic anhydride instead of the phthalic anhydride.

2. Heat the mixture to 80 to 100º C for 2 hours
3. Cool the mixture to ambient temperature
4. Quench the mixture with ice-water containing enough sulfuric acid to make the whole mixture acidic. This mixture will be hydrogen phthalate or succinate, either in the form of oil or a crystalline solid. If the mixture is oil, treat it with acetone and/or use crystal initiation techniques if necessary to crystallize it
5. Filter, wash and then dry the mixture. The result is hydrogen phthalate with free carboxyl functions
6. Use the kits B1, B2 , B3 and B4 and follow instructions under "How to use kits"

Pre-processing of Amino Acids (Amphoteric Racemate)

Amino acids exist in Zwitter ionic structure. A synthetic amino acid is primarily resolved using one of two types of methods:

a) Protection of Carboxylic Group using Esterification

The carboxyl end of the molecule can be protected by esterification followed by diastereomeric salt formation of free amine function and needs screening kits made up of chiral acids. Many racemic alpha-amino acids have been successfully resolved by preparing isobutyl or benzyl esters.

Steps:

Add a sufficient amount of dilute HCL to the racemate to dissolve it and bring the pH to 3

1. Cool the mixture to 0 to 2 º C
2. Esterify by adding (1:1.2 ratio) of isobutyl or benzyl ester
3. Heat the mixture to 100 º C and then cool it to 0 to 5 º C
4. Decrease the acidity to pH-7 by adding NaOH.
5. Use the kits A1, A2, A3 and A4 and follow instructions under "How to use kits" to get the diastereomeric salt
6. After having identified the ideal candidate vial, you should then remove the ester group introduced in step 3 under mild hydrolysis conditions and verify that no racemization occurs

b) Protection of Amino Group using Formylation

The carboxylic group can then be screened with the ChiroSolv kits B1, B2, B3. B4 After having identified the ideal candidate vial, you should then remove the formyl group under mild hydrolysis conditions and verify that no racemization occurs.

Steps:

1. Add a sufficient amount of NaOH solution to the racemate to dissolve it and bring the pH to 10
2. Cool the mixture to 0 to 2 º C
3. Formylate by adding (1:1.2 ratio) of Trithyl Orthoformate
4. Heat the mixture to 100 º C and then cool it to 0 to 5 º C
5. Increase the acidity to Ph-4 by adding Hydrochloric or sulfuric acid
6. Use the kits B1, B2, B3 and B4 & follow instructions under "How to use kits"
7. After having identified the ideal candidate vial, you should then remove the formyl group introduced in step 3 under mild hydrolysis conditions and verify that no racemization occurs

Pre-processing of Aldehydes and Ketones

In order to be resolved by salt formation, aldehydes and ketones must be transformed into either acidic or basic derivatives.

a) Acidic derivatives

Reagents like 4-sulfonylphenylhydrazine, 4-(4-carboxyphenyl) semicarbarzone, 4-hydrazinobenzoic acids (para/meta), Oxalic acids monohydrazide can be used. These
salts can then be resolved by chiral bases.

Steps:

1. Treat the racemic aldehyde or ketone in minimum amount of methanol
2. Cool the mixture to 0 to 5 º C
3. Add one of the above mentioned reagents. The result is a crystalline protected amino acid
4. Isolate the protected amino acid using filtration or centrifusion
5. Use the kits B1, B2, B3, B4 and follow instructions under "How to use kits"

b) Basic derivatives

Carbonyl can be converted into enamine using secondary amines; which can then be resolved by chiral acids. Alternatively, carbonyl is treated with sodium bisulphite and resulting acid function is reacted with chiral amines, and resulting diastereomers can be separated by crystallization.

Steps:

1. Treat the racemic aldehyde or ketone in minimum amount of methanol
2. Cool the mixture to 0 to 5 º C
3. Add secondary amine like pyrrolidene or piperidene. Alternatively you can add Sodium Bisulphite. The result is a crystalline protected amino acid
4. Isolate the protected amino acid using filtration or centrifusion

5. Use the kits A1, A2, A3, A4 for enamine derivative or B1, B2, B3, B4 for bisulphite derivative and follow instructions under “How to use kits”

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