AGENCOURT® CLEANSEQ®

Dye-Terminator Removal

Please refer to http://www.agencourt.com/technical for updated protocols and refer to MSDS instructions http://www.beckmancoulter.com/customersupport/msds/msds.asp when handling or shipping any chemical hazards.
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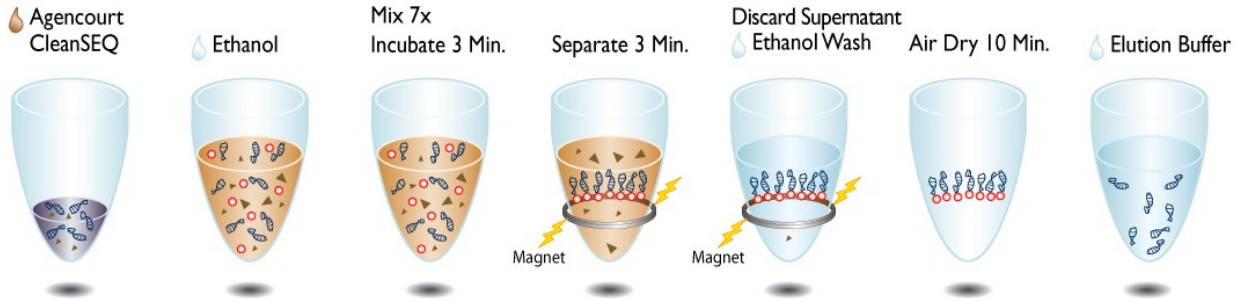
Introduction

Agencourt CleanSEQ is a SPRI® (Solid Phase Reversible Immobilization) magnetic bead-based sequencing purification system with a simple three-step protocol. Its flexible, simple, and automation compatible format is the preferred purification system of many genomic research facilities. The Agencourt CleanSEQ method can be performed directly in the thermal cycling plate and requires no centrifugation or filtration. The system efficiently purifies sequencing products to deliver superior quality sequencing data. Application Notes for Agencourt CleanSEQ can be found at http://www.agencourt.com/technical/.

Agencourt has developed optimized Agencourt CleanSEQ protocols for each of the common sequencing dye sets. This protocol provides instructions for ABI and Beckman sequencing chemistries (see Table of Contents). For ET Terminator users, a *CleanSEQ for MegaBACE** protocol is available at http://www.agencourt.com/technical/.

Process Overview

1. Bind sequencing extension products to magnetic beads, then separate on magnet plate
2. Wash beads to remove unincorporated dyes, nucleotides, salts, and other contaminants
3. Elute DNA using aqueous buffer



Kit Specifications

The amount of CleanSEQ used per sequencing reaction depends on the sequencing chemistry and plate format. Please refer to the charts below to determine how many cleanups each kit can perform

Table 1 ABI BigDye*Terminator

	8 mL A29151 *000121*	***50 mL A29154*** *000136*
96 Well Format	800	5000
384 Well Format	1600	10000

Table 2 Beckman Coulter CEQ DTCS

Reaction Volume	***8 mL A29151*** *000121*	***50 mL A29154*** *000136*
10 µL	800	5000
20 µL	400	2500

Materials Supplied in the Kit

Agencourt CleanSEQ Solution

- Store at 4°C upon arrival, for up to 6 months.
- Mix Agencourt CleanSEQ well before using.
- DO NOT FREEZE.

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

Agencourt CleanSEQ for ABI BigDye Terminator[1]

Materials Supplied by the User:

Consumables & Hardware:

- **Agencourt SPRIPlate® Magnetic Plate:**
  For 96 well format: Agencourt SPRIPlate 96R Ring Magnet Plate
  Beckman #A29164/ Agencourt #000219
  For 384 well format: Agencourt SPRIPlate 384 Magnet Plate
  Beckman #A29165/ Agencourt #000222
- **Reaction Plate:**
  For 96 well format: 96 Well Cycling Plate; Suggested ABGene #AB-1000 or AB-1400, http://www.abgene.com/
  For 384 well format: 384 Well Hardshell Cycling Plate; Suggested ABGene Diamond #AB-1111, http://www.abgene.com/
- **Multichannel pipettes for 10 µL and 100 µL volumes**
- ***Optional*** **Agencourt Direct Inject Magnet** (eliminates final transfer step)
  For 96 well format: Agencourt 96-Well Direct Inject Plate
  Beckman #A29173/ Agencourt #000596
  For 384 well format: Agencourt 384 Direct Inject Plate
  Beckman #A29166/ Agencourt #000296

Reagents:

- **85% Ethanol made from non-denatured ethanol** [American Bioanalytical product # AB-00138, http://www.americanbio.com/]
  - Make 25 mL of 85% ethanol per 96 well plate
  - Ethanol is used for precipitation in the CleanSEQ protocol, so it is critical that the 85% ethanol has been made fresh. To avoid loss of product, make only as much as will be used in 1-3 days and store in a tightly capped container. [American Bioanalytical product AB00138; http://www.americanbio.com/]
- **Elution Buffer: Reagent grade water or 0.1mM EDTA (pH 8.0)**
  - The optimal elution buffer will vary depending on dye chemistry and reaction conditions. See individual protocols for details.

[1] Agencourt CleanSEQ can be used with all BigDye Terminator versions (1.1 and 3.1).

ABI Big Dye Terminator 96 Well Format:

1. **Shake Agencourt CleanSEQ to fully resuspend the magnetic beads before using.**

   The reagent should appear homogenous and consistent in color.

2. **Add 10 µL of Agencourt CleanSEQ to each sample.**

   Use 10 µL of Agencourt CleanSEQ is used regardless of the sequencing reaction volume.

3. **Add 85% ethanol to each sample according the table below. Pipette mix 7 times or until the solution is homogenous.**

   Ethanol floats to the top of the sample, while the Agencourt CleanSEQ sinks to the bottom. It is very important to mix the layers well in order to completely bind the sequencing products to the magnetic beads.

Agencourt CleanSEQ 96 for BigDye Terminator	
Sequencing ReactionVolume (µL)	**Volume of 85% Ethanol (µL)**
5	31
10	42
15	52
20	62
25	73

   For other sample volumes, use the calculation below or use

   the CleanSEQ Calculator at http://www.agencourt.com/technical/**:**

   Volume of 85% Ethanol = 2.077 x (10 µL + Sample Volume)

4. **Place the sample plate onto an Agencourt SPRIPlate 96R for 3 - 5 minutes or until solution is clear.**

   The magnetic beads will form a ring or crescent on the side of the well.

5. **Aspirate the cleared solution (supernatant) from the plate and discard.**

   This step must be performed while the plate is situated on the magnet. To avoid disturbing the beads, place the pipette tip at the bottom of the well when aspirating. Remove as much supernatant as possible as it contains excess fluorescent dye and contaminants.

6. **Dispense 100 µL of 85% ethanol into each well. Wait at least 30 seconds to allow the beads to resettle before continuing to the next step.**

   This step should be performed while the plate is situated on the magnet. It is not necessary to mix or resuspend the beads during this step.

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

7. **Completely remove the ethanol and discard.**

   This step must be performed while the plate is situated on the magnet. Place the pipette tip at the bottom of the well when aspirating to avoid disturbing the beads. Remove as much ethanol as possible, as it contains excess fluorescent dye and contaminants.

8. **Repeat steps 6 – 7 for a total of two 85% ethanol washes.**

9. **Let the samples air-dry for 10 minutes at room temperature.**

   The sample plate can be situated on or off the magnet while drying. Note: Excessive drying can lead to degradation of the fluorescent dye.

10. **Add 40 µL of elution buffer (see chart below) and incubate the plate for 5 minutes at room temperature to elute.** ***If using the Agencourt 96 Direct Inject Magnet Plate[2] (eliminates final plate transfer), elute with 70-80 µL elution buffer.***

    Elution of the sequencing products is rapid. It is not necessary for the beads to go back into solution for complete recovery. Do not denature samples prior to loading on capillary sequencers.

    The suggested elution buffers are 0.1mM EDTA[3] (pH 8.0) or reagent grade water. Water is used to give maximum signal, while EDTA is used to lower the signal in cases where the signal is too strong. The appropriate elution buffer will vary depending on the sensitivity of the sequencing detector, the amount of BigDye used per sequencing reaction and the type of template. Use the following table as a general guideline for choosing an elution buffer.

	ABI 3100 / 3130	ABI 3700	ABI 3730
>2 µL BigDye with PCR Products	0.1mM EDTA[3]	0.1mM EDTA[3]	0.1mM EDTA[3]
<2 µL BigDye with PCR Products	0.1mM EDTA[3]	DiH_2O	0.1mM EDTA[3]
>2 µL BigDye with Plasmids	0.1mM EDTA[3]	DiH_2O	0.1mM EDTA[3]
<2 µL BigDye with Plasmids	DiH_2O	DiH_2O	DiH_2O

    For ABI 3100 users or other users currently eluting in formamide: Please see the application note on "Agencourt CleanSEQ on the ABI 3100"; http://www.agencourt.com/technical.

11. **Allow the sample plate to separate on the magnet for 3-5 minutes or until solution is clear. Transfer 35 µL of the clear sample into a new plate for loading on the detector.** ***If using the Agencourt 96 Direct Inject Magnet Plate, do not transfer.***

    Leave 5 µL - 10 µL of liquid behind to prevent transfer of beads into the final plate. Residual beads can interfere with injection, causing late starts or failed injections. If this occurs, simply re-transfer the samples away from the beads and re-inject.

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[2] *Refer to Direct Inject Protocol at http://www.agencourt.com/technical/; Beckman #A29173/ Agencourt #000596*
[3] Use 0.05mM EDTA in place of 0.1mM EDTA when using Direct Inject Magnet.

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

Seal samples and store at 4°C, for up to 24 hours, prior to loading. If samples will not be loaded within 24 hours, store at -20°C. Samples can be kept at -20°C for at least 1 month.

ABI BigDye Terminator 384 Well Format:

1. **Shake Agencourt CleanSEQ to fully resuspend the magnetic beads before using.**

   The reagent should appear homogenous and consistent in color.

2. **Add 5 µL of Agencourt CleanSEQ to each sample.**

   Use 5 µL of Agencourt CleanSEQ regardless of the sequencing reaction volume.

3. **Add 85% ethanol to each sample according to the table below. Pipette mix 7 times or until the solution is homogenous throughout each well.**

   Ethanol floats to the top of the sample, while the CleanSEQ sinks to the bottom. It is very important to mix the layers well in order to completely bind the sequencing products to the magnetic beads.

Agencourt CleanSEQ 384 for ABI BigDye Terminator	
Sequencing Reaction Volume (µL)	**Volume of 85% Ethanol (µL)**
5	14.3
10	21.4
15	28.6

   For other sample volumes, use the calculation below or use

   the CleanSEQ Calculator at http://www.agencourt.com/technical/**:**

   Volume of 85% Ethanol = 1.428 x (5 µL + Sample Volume)

4. **Place the sample plate on an Agencourt SPRIPlate 384 for 2 - 3 minutes or until solution is clear.**

   The magnetic beads will be pulled to the side of the well.

5. **Aspirate the cleared solution (supernatant) from the plate and discard.**

   This step must be performed while the plate is situated on the magnet. To avoid disturbing the beads, place the pipette tip at the bottom of each well when aspirating. Remove as much supernatant possible as it contains excess fluorescent dye and contaminants.

6. **Dispense 30 µL of 85% ethanol into each well then pipette mix 7 times to wash the beads. Wait for at least 30 seconds to allow the beads to resettle before continuing to the next step.**

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

This step should be performed while the plate is situated on the magnet.

7. **Completely remove the ethanol and discard.**

   This step must be performed while the plate is situated on the magnet. Place the pipette tip at the bottom of the well when aspirating to avoid disturbing the beads. Remove as much ethanol as possible, as it contains excess fluorescent dye and contaminants.

8. **Repeat steps 6 – 7 for a total of two 85% ethanol washes.**

9. **Let the samples air-dry for 10 minutes at room temperature.**

   The sample plate can be situated on or off the magnet while drying. *Note: Excessive drying can lead to degradation of fluorescent dye.*

10. **Add 15 - 30 µL of elution buffer (see chart below) and incubate the plate for 5 minutes at room temperature to elute.**

    Elution of the sequencing products is rapid. It is not necessary for the beads to go back into solution for complete recovery. Do not denature samples prior to loading on capillary sequencers.

    The suggested elution buffers are 0.1mM EDTA (pH 8.0) or reagent grade water. Water is used to give maximum signal, while EDTA is used to lower the signal in cases where the signal is too strong. The appropriate elution buffer will vary depending on the sensitivity of the sequencing detector, the amount of BigDye used per sequencing reaction and the type of template. Use the following table as a general guideline for choosing an elution buffer.

	ABI 3100 / 3130	ABI 3700	ABI 3730
>2 µL BigDye with PCR Products	0.1mM EDTA	0.1mM EDTA	0.1mM EDTA
<2 µL BigDye with PCR Products	0.1mM EDTA	DiH_2O	0.1mM EDTA
>2 µL BigDye with Plasmids	0.1mM EDTA	DiH_2O	0.1mM EDTA
<2 µL BigDye with Plasmids	DiH_2O	DiH_2O	DiH_2O

11. **Allow the sample plate to separate on the magnet for 3-5 minutes or until solution is clear. Transfer clear sample into a new plate for loading on the detector.** ***If using the Agencourt 384 Direct Inject Magnet Plate[4], do not transfer.***

    Leave 2 - 5µL of liquid behind to prevent transfer of bead into the final plate. Residual beads can interfere with injection, causing late starts or failed injections. If this occurs, simply re-transfer the samples away from the beads and re-inject.

    Seal samples and store at 4°C, for up to 24 hours, prior to loading. If samples will not be loaded within 24 hours, store at -20°C. Samples can be kept at -20°C for at least 1 month.

[4] The Agencourt 384 Direct Inject Plate eliminates the final plate transfer step for ABI 3100/3130 and 3730. Beckman #A29166/ Agencourt #000296; http://www.agencourt.com/

Beckman Coulter CEQ DTCS

Materials Supplied by User:

Consumables & Hardware:

- **Agencourt SPRIPlate 96R Ring Magnet Plate**; Beckman #A29164/ Agencourt #000219
- **Plate Seals**: Adhesive or heat, MUST withstand Isopropanol [for example: ABgene #AB0558; http://www.abgene.com/]
- **Multichannel pipettes for 20 µL and 200 µL volumes**
- **Beckman Coulter CEQ sample plate**; Beckman #609801 http://www.beckmancoulter.com/

Reagents:

- **73% Isopropanol** made from 100% Isopropanol: Sigma # I9516-500ML
  - Isopropanol is used for precipitation in the CleanSEQ protocol, so it is critical that the 73% Isopropanol has been made fresh. To avoid loss of product, make only as much as will be used in 1-3 days and store in a tightly capped container.
- **Sample Loading Solution (SLS)**: Supplied with Beckman Coulter Dye Terminator Cycle Sequencing Quick Start Kit; Beckman #608120, http://www.beckmancoulter.com/
- **Beckman Stop Solution Components**:
  - **3M Sodium Acetate pH5.2** - Sigma # S7899
  - **100mM Na_2-EDTA pH 8.0**, prepared from 0.5 M Na_2-EDTA - Sigma # E7889
  - **Glycogen**: Supplied with Beckman Dye Terminator Cycle Sequencing Quick Start Kit #608120) http://www.beckmancoulter.com/]

Beckman Coulter CEQ DTCS 96 Well Format:

This CleanSEQ protocol is optimized for use with 1X DTCS Sequencing Reactions. For best results, follow the reaction setup instructions provided in the DTCS Quickstart Kit # 608120:

- Pre-Treat templates with heat, setup 1X reactions[5], and thermal cycle according to Beckman recommendations.

[5] For this and other dye dilution reactions please refer to Biotechniques 32:24-28 (Jan 2002).

1. **Add 0.55 µL of Dilute Stop Solution (recipe below) for every 1ul of Sequencing Reaction Volume. For example: Add 5.5 µL of Dilute Stop Solution to a 10 µL Sequencing Reaction.**

   Warning: Samples will be difficult to elute later in the protocol if Stop Solution is not added.

   Use the following equation to calculate how much Dilute Stop Solution to make:

   (0.55 x Reaction Volume x Number of Reactions) + 50 µL Dead Volume

Dilute Stop Solution Components	Per 1 µL Dilute Stop Solution	100 µL Dilute Stop Solution (8 x 10 µL Reactions)	600 µL Dilute Stop Solution (96 x 10 µL Reactions)
H_2O	0.5 µL	50 µL	300 µL
3M Sodium Acetate pH 5.2	0.2 µL	20 µL	120 µL
100 mM Na_2-EDTA pH 8.0	0.2 µL	20 µL	120 µL
20 mg/mL glycogen (in DTCS Kit)	0.1 µL	10 µL	60 µL

2. **Shake Agencourt CleanSEQ to fully resuspend the magnetic beads before using.**

   The reagent should appear homogenous and consistent in color.

3. **Add 1 µL Agencourt CleanSEQ for every 1 µL of Sequencing Reaction Volume (disregarding the volume of Stop Solution added in Step 1) to each well of the reaction plate.**

   For example: Add 10 µL of Agencourt CleanSEQ® to a 10 µL Sequencing Reaction (disregarding the 5.5 µL Stop Solution added in Step 1).

4. **Add 73% isopropanol to each sample according to the table below. Pipette mix 10 times, or seal well and vortex the plate for 30 seconds, to thoroughly mix all reagents.** ***Note for Vortexing: Not all adhesive plate seals are resistant to isopropanol!***

   Isopropanol floats to the top of the sample, while the Agencourt CleanSEQ sinks to the bottom. It is very important to mix the layers well in order to completely bind the sequencing products to the magnetic beads.

Agencourt CleanSEQ® 96 for Beckman Coulter CEQ DTCS	
Sequencing Reaction Volume (µL)	**Volume of 73% Isopropanol (µL)**
10	55
20	110

For other sample volumes, use the calculation below:

Volume of 73% Isopropanol = 5.5 x (Sample Volume)

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

5. **Incubate the samples off the magnet for a minimum of 10 minutes to bind the extension products.**

   Longer incubation times will slightly increase the signal of all bases. Shorter incubation times may decrease the signal.

6. **Place the sample plate onto an Agencourt SPRIPlate 96R for 3 - 5 minutes or until solution is clear.**

   The magnetic beads will form a ring or crescent on the side of the well.

7. **Aspirate cleared solution (supernatant) from the plate and discard.**

   This step must be performed while the plate is situated on the magnet. Place the pipette tip at the bottom of the well when aspirating to avoid disturbing the beads. Remove as much of the supernatant as possible, as it contains excess dye and contaminants.

8. **Dispense 200 µL 73% isopropanol to each sample. Incubate for 3 minutes at room temperature before continuing to the next step.**

   This step should be performed while the plate is situated on the magnet. It is not necessary to mix or resuspend the beads during this step.

9. **Completely remove the isopropanol and discard.**

   This step must be performed while the plate is situated on the magnet. Place the pipette tip at the bottom of the well when aspirating to avoid disturbing the beads. Remove as much isopropanol as possible, as it contains excess dye and contaminants

10. **Repeat steps 8-9 for a total of two isopropanol washes.**

11. **Let the samples air-dry for 10 - 20 minutes at room temperature. Do not overdry!**

    The sample plate can be situated on or off the magnet while drying. Note: Allowing samples to dry for longer than 30 minutes may result in lowered signal due to less efficient elution.

12. **With the plate off of the magnet, add 40 µL Beckman Sample Loading Solution (formamide) to each well. Pipette mix 10 - 12 times, or seal and vortex briefly, until beads are homogeneously resuspended in SLS.**

    Beads must go back into solution. Continue mixing until the beads have been thoroughly resuspended. If Stop Solution was not used, the beads will be very difficult to resuspend.

    Note: Recovery is dependent on the quality of the SLS. SLS that has been frozen and thawed multiple times could result in partial elution and lead to decreased signal intensity and/or quality of the sequencing samples.

13. **Allow the sample plate to separate on the magnet for 3-5 minutes or until solution is clear. Transfer 35 µL of the clear sample into a new plate for loading on the CEQ.**

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

Leave 5 µL - 10 µL of liquid behind to prevent transfer of beads into the final plate. Some bead carry-over is not detrimental, but care should be taken to minimize it. If significant carry-over occurs, simply dispense sample back into the well, allow the beads 1-3 minutes to resettle and retry transfer step.

14. **Centrifuge briefly to eliminate air bubbles. Overlay the 35 µL with 1 drop of mineral oil provided in Beckman Coulter Kit# 608120 and run the reactions on CEQ sequencer as normal.**

Troubleshooting Guide (ABI BigDye Terminator Only):

Symptom	Reason	Solution
Dye Blobs (dye peaks usually at 70 and 100 bases)	Insufficient Supernatant Removal	Check the plate visually and make sure supernatant and ethanol washes are removed completely; aspirate a second time if necessary
	Too much BigDye	Use less BigDye per sequencing reaction; contact support@agencourt.com if needed
	Formamide elution	Elute in water or EDTA; see protocol and Application note: CleanSEQ® on ABI 3100 http://www.agencourt.com/technical/
	Ethanol concentration too high	Add correct amount of ethanol for sequencing reaction volume and make sure ethanol was made up correctly
Low signal (signal intensity is similar to intensity of background noise)	Insufficient mix	Make sure appropriate number of mixes are performed; visually inspect wells to make sure reactions look homogeneous
	Bead loss	Make sure no beads are aspirated during supernatant removal; dispense back supernatant and attempt again with a smaller volume after beads have re-settled if this happens
	Low ethanol concentration	Make sure ethanol and stock bottle is fresh; make sure correct volume is added for sequencing reaction volume; measure ethanol and water in separate containers before combining
Overload (Signal intensity is extremely high, may appear as flat peaks in electropherogram)	Too much BigDye	Use less BigDye per sequencing reaction; contact support@agencourt.com if needed Transfer only part of the eluant for loading If signal is only high in the beginning of the electropherogram and then rapidly decreases, elute in EDTA (see protocol)