Nucleics

dLUTE SEQ™

Users manual

Version 2.0

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Introduction

The dLUTE SEQ™ DNA sequencing system allows nanolitre-scale DNA sequencing reactions to be performed using microlitre-scale equipment and protocols.

The dLUTE SEQ system utilizes an optimized formulation as a reaction carrier to allow the easy manipulation of sub-microlitre volumes. A large volume of the dLUTE SEQ formulation is initially combined with small volumes of DNA sequencing components (i.e. sequencing chemistry premix, primer and template) to form a stable mixture. This mixture can be easily handled and aliquoted with standard lab or robotic pipetting systems. The mixture is then induced to separate by heat into a small nanolitre-scale reaction phase and a larger, inert carrier phase. After phase separation the inert carrier phase of the dLUTE SEQ formulation overlays the aqueous reaction phase and prevents evaporation. As a consequence dLUTE SEQ reactions can be performed in open reaction vessels. Figure 1 shows how a dLUTE SEQ reaction compares to a standard DNA sequencing reaction.

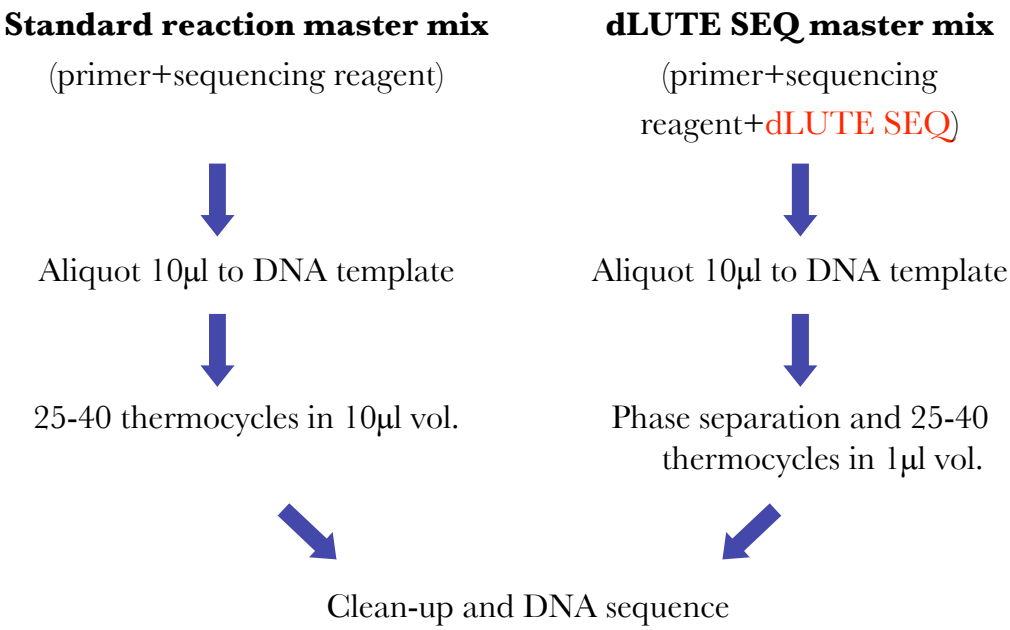

Figure 1. An overview of the dye terminator DNA sequencing process for standard and dLUTE SEQ reactions.

The dLUTE SEQ systems can be used with all DNA cycle sequencing chemistries including Applied Biosystems' BigDye™ and Amersham Biosciences' ET Terminator chemistries.

dLUTE SEQ system components

dLUTE SEQ Sequencing Formulation

The *dLUTE SEQ Sequencing Formulation* (PURPLE capped tube) should be ***stored at 4˚C*** and is stable for at least 3 months. Before use ensure the dLUTE SEQ formulation is homogeneous by vortexing for 10 to 30 seconds.

dLUTE SEQ Phase Indicator

The *dLUTE SEQ Phase Indicator* (YELLOW capped tube) is used to colour the reaction phase and it does not interfere with the DNA sequencing reaction. The *dLUTE SEQ Phase Indicator* should be ***stored at -20˚C***.

dLUTE SEQ DNA sequencing protocols

Four protocols are provided for the 1ml dLUTE SEQ system (NUC-20-1) and the 25 ml dLUTE SEQ system (25 ml; NUC-20-25). Protocol #1 is designed for experiments where the same primer is used for all templates (e.g. sequencing of inserts cloned in a common vector). Protocol #2 is designed for experiments where DNA template specific primers are used (e.g. primer walking). The critical steps are indicated in bold.

Protocol #1 (dLUTE SEQ + primer) in 96 well trays

1. Add 3 – 15ng/1000 bp of DNA template to each 96 plate well (i.e. for 5000 bp templates add 15-75ng total). **!! Ensure all sample liquid is in the bottom of the plate wells !!**
2. Dry the DNA template. This should take approximately 20 minutes at 80˚C (or overnight at room temperature) for 3µl to 5µl of DNA template. **!! Ensure all samples are completely dry before proceeding. Don't use templates that contain EDTA !!**
3. Vortex the *dLUTE SEQ DNA Sequencing Formulation* for 10 – 30 seconds. **!! Ensure that the formulation is opaque white and homogeneous !!**
4. Add 2.5 pmol of primer per reaction to the *dLUTE SEQ* mixture (i.e. 100 reactions require a total of 250pmol). **!! The required primer should be in 5µl !!**
5. Add 5µl of *dLUTE SEQ Phase Indicator*.
6. Add 40µl of sequencing chemistry premix reagent (e.g. BigDye™). **!! dLUTE SEQ mixture must be used within four hours of addition of sequencing premix !!**
7. Mix solutions by shaking tube up and down vigorously for 10 second. The dLUTE SEQ formulation should become pink. **!! Ensure mixture is evenly pink with no white zones !!**
8. Add 2.5 – 10µl aliquots of the *dLUTE SEQ* mixture to each PCR plate well containing dried template DNA. Spin plate briefly (~30 s) to ensure that the dLUTE mixture is at the bottom of the wells. **!! dLUTE SEQ reactions below 5µl should be performed in 384-well plates only !!**
9. Thermocycle using standard cycle sequencing reactions conditions with a 75˚C for 3 minute preincubation step (eg 75˚C for 3 min, 45x {95˚C 10 s, 50˚20 s, 60˚C 4 min}). **!! The 75˚C step is critical to ensure separation on the insert and aqueous phases before the reaction is heated to 95˚C. Failure to include this step will result in inactivation of the DNA polymerase !!**
10. Perform sequencing reaction clean-up using the supplied *dLUTE SEQ Alcohol Precipitation Protocol* or *dLUTE SEQ Alcohol/Isopropanol Precipitation clean-up for 384-well plate*. Alternatively, if using a compatible commercial clean-up kit, add 10µl of 1x sequencing reaction dilution buffer (2 mM $MgCl_2$, 80mM Tris, pH9) to each reaction and follow the kit's protocol without modification.

!! Do not perform standard ethanol precipitation clean up on dLUTE SEQ reactions !!

Protocol #2 (dLUTE SEQ only) in 96 well trays

1. Add 3 – 15ng/1000 bp of DNA template to each 96 plate well (i.e. for 5000 bp templates add 15-75ng total). **!! Ensure all sample liquid is in the bottom of the plate wells !!**
2. Add 2.5pmol of primer to each plate well. **!! Ensure all sample liquid is in the bottom of the plate wells !!**
3. Dry the DNA template. This should take approximately 20 minutes at 80˚C (or overnight at room temperature) for 3µl to 5µl of DNA template. **!! Ensure all samples are completely dry before proceeding. Don't use templates that contain EDTA !!**
4. Vortex the *dLUTE SEQ DNA Sequencing Formulation* for 10 – 30 seconds.**!! Ensure that the formulation is opaque white and homogeneous !!**
5. Add 10µl of *dLUTE SEQ Phase Indicator*.
6. Add 40µl of sequencing chemistry premix reagent (e.g. BigDye™). **!! dLUTE SEQ mixture must be used within four hours of addition of sequencing premix !!**
7. Mix solutions by shaking tube up and down vigorously for 10 second. The dLUTE SEQ formulation should become pink. **!! Ensure mixture is evenly pink with no white zones !!**
8. Add 2.5 – 10µl aliquots of the *dLUTE SEQ* mixture to each PCR plate well containing dried template DNA. Spin plate briefly (~30 s) to ensure that the dLUTE mixture is at the bottom of the wells. **!! dLUTE SEQ reactions below 5µl should be performed in 384-well plates only !!**
9. Thermocycle using standard cycle sequencing reactions conditions with a 75˚C for 3 minute preincubation step (eg 75˚C for 3 min, 45x {95˚C 10 s, 50˚20 s, 60˚C 4 min}). **!! The 75˚C step is critical to ensure separation on the insert and aqueous phases before the reaction is heated to 95˚C. Failure to include this step will result in inactivation of the DNA polymerase !!**
10. Perform sequencing reaction clean-up using the supplied *dLUTE SEQ Alcohol Precipitation Protocol* or *dLUTE SEQ Alcohol/Isopropanol Precipitation clean-up for 384-well plate*. Alternatively, if using a compatible commercial clean-up kit, add 10µl of 1x sequencing reaction dilution buffer (2 mM $MgCl_2$, 80mM Tris, pH9) to each reaction and follow the kit's protocol without modification.

!! Do not perform standard ethanol precipitation clean up on dLUTE SEQ reactions !!

dLUTE SEQ Alcohol Precipitation Clean-up

The dLUTE SEQ reagent is not compatible with the standard ethanol precipitation clean up methods. The following protocol is an alternative protocol that provides high quality data. This protocol is suitable for reactions performed in 96-well plates.

1. Add 150µl of *dLUTE SEQ Precipitation Solution* (35% (vol/vol) 1-butanol: 65% (vol/vol) absolute ethanol) to each well. **!! Use absolute (100%) ethanol only !!**
2. Pipette up-and-down 12 times. **!! Ensure the aqueous phase is completely dispersed in the *dLUTE SEQ Precipitation Solution !!***
3. Centrifuge for 30 min at room temperature and maximum speed (min. 3,500 g)
4. Discard the supernatant by inverting the plates onto a paper towel.
5. Place the inverted plate with paper towel in the centrifuge.
6. Centrifuge at 500 x g for 1 min at room temperature.
7. Add 150µl of *dLUTE SEQ Wash Solution* (65% absolute ethanol: 35% 0.1mM EDTA).
8. Centrifuge at maximum speed (min. 3,500 x g) for 10 min at room temperature.
9. Without disturbing the pellet, discard the supernatant by inverting the wells on to a paper towel.
10. Add 150µl of *dLUTE SEQ Wash Solution* (65% absolute ethanol: 35% 0.1mM EDTA).
11. Centrifuge at maximum speed (min. 3,500 x g) for 10 min at room temperature.
12. Without disturbing the pellet, discard the supernatant by inverting the wells on to a paper towel.
13. Place the inverted plate with paper towel in the centrifuge
14. Centrifuge at 500 x g for 1 min at room temperature
15. Dry pellet and resuspend in loading solution

dLUTE SEQ Alcohol Precipitation clean-up for 384-well plates

The dLUTE SEQ reagent is not compatible with standard ethanol precipitation clean-up methods. The following protocol is an alternative protocol for use with 384-well plates that have a maximum volume of 35µl per well.

1. Add 25µl of *dLUTE SEQ precipitation solution* (65% (vol/vol) 1-butanol, 35% (vol/vol) absolute ethanol). **!! Use absolute (100%) ethanol only !!**
2. Pipette up-and-down 12 times. **!! Ensure the aqueous phase is completely dispersed in the dLUTE SEQ precipitation solution !!**
3. Centrifuge for 30 min at room temperature and maximum speed (min. 3,500 g).
4. Place the inverted plate on a paper towel in the centrifuge.
5. Centrifuge at 100 x g for 1 min at room temperature.
6. Add 30µl of *dLUTE SEQ wash solution* (60% absolute ethanol: 40% 0.1 mM EDTA) and mix by pipetting up-and-down.
7. Centrifuge for 10 min at room temperature and maximum speed (min. 3,500 g).
8. Place the inverted plate on a paper towel in the centrifuge.
9. Centrifuge at 100 x g for 1 min at room temperature.
10. Add 30µl of *dLUTE SEQ wash solution* (60% absolute ethanol: 40% 0.1 mM EDTA) and mix by pipetting up-and-down.
11. Centrifuge for 10 min at room temperature and maximum speed (min. 3,500 g).
12. Place the inverted plate on a paper towel in the centrifuge.
13. Centrifuge at 100 x g for 1 min at room temperature.
14. Dry pellet and resuspend in loading solution.

dLUTE SEQ Isopropanol Precipitation clean-up for 384-well plates

The dLUTE SEQ reagent is not compatible with the standard ethanol precipitation clean-up methods. The following protocol is an alternative protocol for use with 384-well plates that have a maximum volume of 35µl per well.

1. Add 25µl of *dLUTE SEQ precipitation solution* (80% (vol/vol) isopropanol, 20% (vol/vol) 1-butanol supplemented with 0.2% (vol/vol) 40 mM magnesium chloride).
2. Pipette up-and-down 12 times. **!! Ensure the aqueous phase is completely dispersed in the dLUTE SEQ precipitation solution !!**
3. Leave at room temperature for 15 min.
4. Centrifuge for 30 min at room temperature and maximum speed (min. 3,000 g).
5. Place the inverted plate on a paper towel in the centrifuge.
6. Centrifuge at 100 x g for 1 min at room temperature.
7. Add 30µl of *dLUTE SEQ wash solution* (60% absolute ethanol: 40% 0.1 mM EDTA) and mix by pipetting up-and-down.
8. Centrifuge for 10 min at room temperature and maximum speed (min. 3,000 g).
9. Place the inverted plate on a paper towel in the centrifuge.
10. Centrifuge at 100 x g for 1 min at room temperature.
11. Add 30µl of *dLUTE SEQ wash solution* (60% absolute ethanol: 40% 0.1 mM EDTA) and mix by pipetting up-and-down.
12. Centrifuge for 10 min at room temperature and maximum speed (min. 3,000 g).
13. Place the inverted plate on a paper towel in the centrifuge.
14. Centrifuge at 100 x g for 1 min at room temperature.
15. Dry pellet and resuspend in loading solution.

Recommendations

DNA Template

dLUTE SEQ reactions require much smaller amounts of DNA template than conventional reactions. Nucleics recommends the use of 3 ng to 15 ng of DNA template per 1000 bp of template (eg. for a 5000 bp template use a total of 15 to 75ng of DNA). **!! The using too much DNA will result in sequencing reaction failure or sub-optimal results !!**

In order to perform the DNA sequencing reaction at a nanolitre-scale volume the DNA template must be in a nanolitre volume. This can be most easily accomplished by adding the DNA template to the reaction tube or plate well in a microlitre volume (i.e. 2 µl to 5 µl) and drying the sample. The sequencing primer may also be added at this stage if desired.

Nucleics recommends the use of DNA templates that are highly purified. dLUTE SEQ is compatible with many commercially available DNA purification kits. The uses of DNA storage buffers containing EDTA should be avoided.

Sequencing primer

The primer amounts given in the protocol are for standard sequencing primers (e.g. M13 forward or reverse sequencing primers). Other primer may require smaller or larger amounts for optimal results.

Reaction volume

The preparation of the dLUTE SEQ mixture can be scaled to the sensitivity of the DNA sequencer used by simply increasing or decreasing the reaction volume. High quality sequencing reactions can be obtained at the 2.5 µl scale (i.e. a reaction phase of 250 nanolitres) using the Applied Biosystems 3700 DNA sequencer. **It is recommended that dLUTE SEQ reactions below 5µl are NOT performed in 96 well plates.**

Thermocycling conditions

Standard sequencing thermocycling condition can be used with dLUTE SEQ, however, an initial 75˚C 3 minute preincubation step is required to separate the inert and aqueous phases. Failure to include this step will cause inactivation of the DNA polymerase due to the high surface area the polymerase is exposed to in the non-separated dLUTE mix.

Handling

The viscosity and fluid properties of the dLUTE SEQ formulation differs from other sequencing solutions. Nucleics recommend using positive displacement with your pipetting equipment to ensure that accurate volumes are manipulated. The dLUTE SEQ formulation has a density of 0.83 g/ml.

Sequencing clean-up

dLUTE SEQ reactions can be purified in the same fashion as normal dye terminator sequencing reactions after the aqueous volume is increased to 10µl (p.5). dLUTE SEQ has been shown to be compatible with the following commercial clean-up systems:

- SEQueky Kleen™ H20 system (BioRad)
- AutoSeq96™ system (Amersham Biosciences)
- Biotin-Strepavidin system (Dynal)
- Wizard MagneSil™ GREEN system (Promega)

Other sequencing clean-up systems may also be compatible with dLUTE SEQ but have not been tested.

Sequencing chemistry dilution

Nucleics does not recommend the use of standard DNA sequencing premix dilutions with dLUTE SEQ. Dilutions of the sequencing chemistry have been found to provide less consistent data. If further reductions in reagent usage are desired, it is recommended that the volumes of the dLUTE SEQ reactions are reduced further (i.e. by performing 2.5 µl dLUTE SEQ reactions).

While the use of sequencing chemistry dilutions with dLUTE SEQ generally provide sub-optimal results, the increased sensitivity of some new machines (such as the ABI3730xl) permits modest dilutions to be used. If performing dilution reactions it is recommended that a 2.5 x Sucrose dilution buffer (200 mM Tris-HCl, pH 9; 5 mM $MgCl_2$, 1.5 M sucrose) is used in the range of 1:1 to 1:3 (i.e. 20µl of BigDye + 20µl of 2.5 x Sucrose dilution buffer or 10µl of BigDye + 30µl of sucrose dilution buffer per ml of dLUTE SEQ formulation).

Technical Support

Nucleics provides expert technical support for the dLUTE SEQ system. Please contact us at:
information@nucleics.com