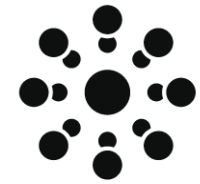

Nucleics

dLUTE SEQ CF™

Users manual

July 2009

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Introduction

The dLUTE SEQ CF™ protocol allow the small reaction volumes to be handled using standard lab equipment. 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.

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

dLUTE SEQ CF Components

dLUTE SEQ CF Sequencing Formulation

The *dLUTE SEQ Sequencing Formulation* (BLUE 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 CF Dilution Buffer

The *dLUTE SEQ CF Dilution Buffer* (GREEN capped tube) is used to dilute the BigDye reagent. The *dLUTE SEQ CF Dilution Buffer* should be ***stored at -20˚C***.

dLUTE SEQ CF Protocol for 96 Well Trays

1. Add 1 µl of template DNA containing 3 – 15ng/1000 bp of DNA template to each 96 plate well (i.e. for 5000 bp templates add 15-75ng total template DNA). **!! Ensure the template liquid is at the bottom of the plate wells. Don't use DNA solutions which contain EDTA greater than 0.1mM!!**

2. Vortex the *dLUTE SEQ DNA Sequencing Formulation* for 10 – 30 seconds. **!! Ensure that the formulation is an opaque white and homogeneous !!**

3. Add 2.5 pmol of primer per reaction to the *dLUTE SEQ* mixture (i.e. 100 reactions require a total of 250pmol). **!! The total required primer for all 96 wells should be in 5µl !!**

4. Add 25µl of *dLUTE SEQ CF Dilution Buffer*. **!! Only use the GREEN capped tube phase indicator solution !!**

5. Add 20µl of DNA sequencing chemistry premix reagent (e.g. BigDye™). **!! The dLUTE SEQ mixture must be used within four hours !!**

6. Mix solutions by hand shaking the dLUTE SEQ formulation vigorously for 15 seconds. You should be able to hear the glass bead inside the tube moving up and down. **!! Do not vortex or mix by pipetting up and down as this will cause the dLUTE SEQ phases to separate!!**

7. Add 10 µl aliquots of the *dLUTE SEQ* mixture to each PCR plate well or tube **containing 1 µl of template DNA**. Spin plate or tube briefly (~30 s at ~ 1000g) to ensure that the dLUTE mixture and template are at the bottom of the wells.

8. Thermocycle using standard cycle sequencing reactions conditions with a 75˚C for 3 minute pre-incubation step (ie 75˚C for 3 min, 45x {95˚C 10 s, 50˚C 20 s, 60˚C 4 min}). It is best to pre-heat the PCR machine and add the plate/tubes once 75˚C is reached. **!! 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 sequencing DNA polymerase !!**

9. Add 10µl of 1x sequencing reaction dilution buffer (2 mM $MgCl_2$, 80mM Tris, pH9) to the aqueous phase (bottom) in each reaction and follow the sequencing clean up kit's protocol without modification. **!! Do not perform standard ethanol precipitation clean-ups on dLUTE SEQ reactions !!**

dLUTE SEQ CF Alcohol Precipitation Clean-up

The dLUTE SEQ CF 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 sequencing loading solution.

Recommendations

DNA Template

dLUTE SEQ CF 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). **!! Using too much DNA will result in sequencing reaction failure or sub-optimal results !!**

Nucleics recommends the use of DNA templates that are highly purified. dLUTE SEQ CF is compatible with many commercially available DNA purification kits. **The uses of DNA storage buffers containing EDTA above 0.1mM 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 dLUTE SEQ CF reaction has been optimised for use with 10 µL of dLUTE and 1 µL of template. **If you wish to use different volumes it is strongly recommended that you use the standard dLUTE SEQ system. If you are using custom primers and can not add a single primer directly to the dLUTE SEQ mix then it is recommended that you add 0.5µl of template and 0.5 µl of primer.**

Thermocycling conditions

Standard sequencing thermocycling condition can be used with dLUTE SEQ CF, however, an initial 75˚C 3 minute pre-incubation step is required to separate the inert and aqueous phases. It is best to allow the PCR machine to reach 75C before the plate/tubes are added. **Failure to include this step will cause inactivation of the sequencing DNA polymerase due to the high surface area that the polymerase is exposed to in the non-separated dLUTE mix.**

Handling

The viscosity and fluid properties of the dLUTE SEQ CF formulation differs from other DNA sequencing dilution solutions. Nucleics recommend pipetting dLUTE using a positive displacement technique **with wide bore pipette tips** to ensure that accurate volumes are manipulated. Alternatively dLUTE can be handled by weight. Before addition of the sequencing reagents **the dLUTE SEQ formulation has a density of 0.83 g/ml.**

Sequencing clean-up

dLUTE SEQ CF 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 CF 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)
- Clean Seq™ (Agencourt). *If you are using the Agencourt system please contact us as we have a dLUTE SEQ protocol optimised for use with Clean Seq.*

Other sequencing clean-up systems may also be compatible with dLUTE SEQ but have not been tested. **If you wish to clean up your reaction using ethanol precipitation we strongly recommend you use the protocol described on p.6 of this manual.**

Sequencing chemistry dilution

Nucleics does not recommend the use of other DNA sequencing dilutions solutions with dLUTE SEQ CF. The reaction has been optimised for a fixed volume and any changes are likely to result is suboptimal or failed reactions.

Technical Support

Nucleics provides expert technical support for the dLUTE SEQ CF system.

Please contact us at: information@nucleics.com