DeepChek®

SANGER SEQUENCING REACTION

V1.2 (RUO)

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24
48

User Guide

Version 1 – Revision 2

For use with Capillary Electrophoresis (CE) instruments

For Research Use Only (RUO). Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of disease.

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123A48 – GTIN 05407007960040

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Document control

Date	Device version	IFU version	Description of change
2022/11/04	A	1.2	• Add legend of tables and figures • Add box numbering information in table 1 • Add figures related to boxes content and disposal • New support website address link
2022/04/22	A	1.1	• Volumes of assay components added for version 24 and 48 reactions (section 1.b) • Changes in the section 5 – Purifying the sequencing reactions

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1. Application

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The ***DeepChek® SANGER SEQUENCING REACTION (RUO)*** is a universal sequencing kit that can be used for all kinds of applications on Sanger sequencing instruments:

- Sequencing of (long range) PCR products with maximum read lengths with standard or long run modules
- Sequencing of plasmid DNA with maximum read length
- Sensitive heterozygote detection with optimized peak heights distribution

2. Summary and explanation of the test

The ***DeepChek® SANGER SEQUENCING REACTION (RUO)*** is a complete kit, based on the trusted Sanger Chain Termination method.

The kit is delivered as a ready-reaction premix, fully optimized for a highly flexible chemistry, designed for all kinds of Sequencing applications, including de novo sequencing and resequencing.

The kit reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on PCR fragments, and on large templates (e.g., BAC clones).

The kit generates data with uniform peak heights and optimized signal balance to produce long and high-quality reads.

a) Workflow

Figure 1: Sanger Sequencing Reaction worklow

b) Assay components

The ***DeepChek® SANGER SEQUENCING REACTION (RUO)*** is provided in two formats:

- 24 reactions (REF 123A24 / GTIN: 05407007960057)
- 48 reactions (REF 123A48 / GTIN: 05407007960040)

Table 1: Components of the DeepChek® SANGER SEQUENCING REACTION assay for models 24 and 48 tests

Label	Volume for 24 Rxn	Volume for 48 Rxn	Color cap	Box number	Storage
PCR Purification	1 x 65 µL	1 x 130 µL	Orange	1	-25°C to - 15 °C
Sequencing Reaction (BDT 3.1)	1 x 260 µL	1 x 520 µL	Pink	1	-25°C to - 15 °C
Sequencing Reaction (Buffer)	1 x 130 µL	1 x 260 µL	Blue	2	+2°C to +8°C
Sequencing Purification (SAM™ Solution)	4 x 1325 µL	10 x 1200 µL	Brown	2	+2°C to +8°C
Sequencing Purification (BDX)	1 x 1300 µL	2 x 1300 µL	Purple	2	+2°C to +8°C

Note: All volumes include 10% overage for pipette error. Do not mix the reagents from different batches.

- Model 123A24

Box 1

	PCR Purification		Sequencing Reaction (BDT 3.1)	

Figure 2: Disposal of the assay components for the DeepChek® SANGER SEQUENCING REACTION model 24 tests – Box 1

Box 2

	Sequencing Reaction (Buffer)		Sequencing Purification (BDX)	
Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)		Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)

Figure 3: Disposal of the assay components for the DeepChek® SANGER SEQUENCING REACTION model 24 tests – Box 2

- Model 123A48

Box 1

	PCR Purification		Sequencing Reaction (BDT 3.1)	

Figure 4: Disposal of the assay components for the DeepChek® SANGER SEQUENCING REACTION model 48 tests – Box 1

Box 2

	Sequencing Reaction (Buffer)		Sequencing Purification (BDX)	Sequencing Purification (BDX)
Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)
Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)	Sequencing Purification (SAM™ Solution)

Figure 5: Disposal of the assay components for the DeepChek® SANGER SEQUENCING REACTION model 48 tests – Box 2

c) Materials required but not provided

- Microliter pipets* dedicated for PCR (0.1-2.5 µL; 1-10 or 1-20 µL; 20-200 µL; 1000 µL)
- Benchtop centrifuge* with rotor for 0.5 mL/1.5 mL reaction tubes (capable of attaining 10,000 rpm)
- Benchtop vortex mixer*
- The product is used with a thermal cycler* for the amplification (PCR) which is a general laboratory use device with associated specific material and with enough thermal ramp rate of 1°C/s, 96 well (0.2 mL standard format), heated lid (100°C), fully programmable in multiple stages and capability to cool down to 4°C at the end of the program.

(*): Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Note:

- Please refer to relevant manufacturer's Instructions for Use (IFU) to proceed with the instrument.
- For the thermal cycler, ramping time is very important. If the thermal ramping time is too fast (>1°/second), poor (noisy) data may result.

d) Reagent storage and handling

The Assay (Box 1) is shipped on dry ice and must be stored at –25°C to –15°C upon receipt. The Assay (Box 2) is shipped at 2°C to 8°C and must be stored at 2°C to 8°C upon receipt.

- All reagents should be thawed completely before use.
- Gently mix and centrifuge the tubes before opening.
- Store all kit components in original containers.
- Use temperature-controlled refrigerators.

These storage conditions apply to both opened and unopened components. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

Expiration dates for each reagent are indicated on the individual component labels. Under correct storage conditions, the product will maintain performance until the expiration date printed on the label.

There are no obvious signs to indicate instability of this product. The operator may remove non-conform assays for research use only. The operator may have a dedicated storage area for conform assays.

e) Starting

- Identify the product.
- Verify the expiration date.
- Verify the latest instruction for use available for the product lot number.
- Verify if the product was used already. If yes, check the remaining tests available.

3. Preparing PCR Products for the Sequencing Reactions

a) Dilution

A common cause of poor Sequencing results is the quality, or the quantity of the template used for the sequencing reaction. The template should be as much as possible free from proteins, RNA, chromosomal DNA, PCR primers, dNTPs, enzymes, buffer components, salts, organic chemicals, and residual detergents.

For setting up the cycle sequencing reaction, use the following guidelines in template quantity.

Too low template results in weak signals and elevated signal-to-noise (S/N) ratios. Too much template results in short reads with overloaded signals.

Table 2: Appropriate template quantities according to the product used for the sequencing reaction

PCR 100-200 bp	1-3 ng
PCR 200-500 bp	3-10 ng
PCR 500-1000 bp	5-20 ng
PCR 1000-2000 bp	10-40 ng
>200 bp	20-50 ng
Plasmid DNA	150-300 ng

In presence of very large PCR bands on the agarose gel or equivalent capillary electrophoresis reagent, e.g. Agilent ScreenTape D1000 and Reagents D1000 for Agilent TapeStation 4150, make dilution of the PCR product before sequencing.

Table 3: General guidance for the dilution step

If the DNA concentration is	Then...
greater than 100 ng	add sterile, deionized water to the sample for a dilution of 1/20
between 60–100 ng	add sterile, deionized water to the sample for a dilution of 1/10
between 30–60 ng	add sterile, deionized water to the sample for a dilution of 1/5
between 10–30 ng	add sterile, deionized water to the sample for a dilution of ½
less than 10 ng	No dilution is recommended here.

1. Open the tube cap.
2. Add sterile, deionized water to the sample for the required dilution amount.
3. **Close the tube cap after each dilution.**
4. Vortex briefly.
5. Pulse-spin at low speed for 5–10 seconds to collect the contents at the bottom of the tube.
6. Keep the diluted reactions on ice.

b) Purifying

For optimum results, purify the PCR product before sequencing by removing dNTPs and primers.

The *PCR purification* reagent is an ultra-fast method that degrades primers and nucleotides (dNTP'S) from PCR products, to make it ready for downstream applications, such as Cycle Sequencing.

Note: Keep *PCR purification* reagent on ice or in a cooling block during the procedure.

1. Mix 5 µl of PCR product with 2 µl of *PCR purification* reagent
2. Place tubes in Thermal Cycler with heated lid, to prevent evaporation
3. Incubate 4 minutes at 37°C to perform enzymatic purification
4. Heat Inactivate 1 minute at 80°C => Hold at 4°C

5. The PCR product is now ready for sequencing. Taking care of the concentration, a further dilution step maybe required.
6. When the purification step has finished, either hold the samples at 10˚C or store at –15 to –25 ˚C before further use.

Note: Optimal template input (ng) in *Sequencing Reaction* Cycle Sequencing reaction can be calculated by dividing the PCR product length (bp) by 50. Example: Use 10 ng of PCR product with a length of 500 bp as template. Close each sample tube cap after each purification.

Once the dilution and purification steps are finished (Templates preparation), each PCR product is now ready for use in DNA sequencing.

4. Performing the Cycle Sequencing Reactions

The PCR products are used as templates in the cycle sequencing reactions, which generate fluorescently labeled DNA size ladders for sequencing. Identify your custom sequencing primers. The operator shall use the Sequencing Reaction (BDT 3.1) (BigDye Terminator v 3.1 chemistry) to perform the cycle sequencing reactions.

a) Primer Quality/Quantity

Always use high quality primers for Cycle Sequencing, as well as for generating PCR template. Most common cause of primer issues is the so-called N-1 artifact, caused by primer solutions that contain partially non-full-length product, causing the typical “n-1 stutter peaks”.

We recommend storing Sequencing primers in a concentration of 3.2 µM (pMol/µL) at -20°C and avoid many freeze-thaw cycles. Use 3-5 pMol sequencing primer per reaction.

b) Reaction Setup

- For Sequencing, use the Sanger sequencing primer (forward and reserve – 3.2µM). One µL of each primer will be used for each sample.
- Prepare the Sequencing reaction according to the following tables.

Table 4: PCR reaction setup for reverse and forward sequencing with the DeepChek® SANGER SEQUENCING REACTION assay

Reagent volume for *Forward* Sequencing	Volume
Sequencing Reaction (BDT 3.1)	4 µL
Sequencing Reaction (Buffer)	2 µL
Template	2 µL
Forward Primer (3.2 µM)	1 µL
Water	11 µL
Total volume	**20 µL**

Reagent volume for *Reverse* Sequencing	Volume
Sequencing Reaction (BDT 3.1)	4 µL
Sequencing Reaction (Buffer)	2 µL
Template	2µL
Reverse Primer (3.2 µM)	1 µL
Water	11 µL
Total volume	**20 µL**

c) Cycle Sequencing Reaction conditions

- **A single PCR thermal cycling program shall be entered in the PCR thermal cycler for the Cycle Sequencing Reaction**
- **To avoid handling errors, identify properly the sequencing primers tubes**
- **Do not expose sequencing primers mixes to light for extended periods**

Once your Cycle Sequencing Reactions for the sequencing primers are ready, please perform the PCR.

1. Review the thermal cycler program for correctness.

Table 5: PCR cycling program

Stage	Temperature (°C)	Time (min:sec)
Initial denaturation	96	01:00
25 cycles	96	0:10
	50	0:05
	60	4:00
Cooling	4	End of program

Ramp rate: 1°C/s

2. Transfer the Cycle Sequencing PCR tubes (that are on ice).
3. Start the thermal cycler.
4. When the program has finished, either hold the samples at 10˚C until you are ready to perform the Sequencing purification (within the next 12 hours) or store at –15 to –25 ˚C.

Note:

- The operator shall use a calibrated thermal cycler for the incubation step.
- The operator shall select the correct tray and retainer assemblies for the tubes and thermal cycler.

5. Purifying the Sequencing Reactions

a) Principle

At the end of the cycle sequencing reactions, each tube contains a fluorescently labeled DNA sequence ladder. The operator shall use the Sequencing Purification reagents which purify the cycle-sequencing reaction by removing unwanted components such as salt ions, unincorporated dye terminators and dNTPs. This prevents their co-injection with your sequencing products.

Two reagents are used, Sequencing Purification (BDX) and Sequencing Purification (SAM Solution). These reagents can be added as a premix or sequentially. Cleanup is complete in under 40 minutes and requires less than 10 minutes of hands-on time.

Important!

When loading plates directly into the capillary electrophoresis instrument, use the BigDye XTerminator Purification Kit run modules specified for your instrument. The run modules are available at www.thermofisher.com/sangerpatches.

These BDX run modules adjust the sample injection height to prevent the capillary array from going into the Sequencing purification *material* at the bottom of the wells, potentially affecting the data.

- Before pipetting, make sure the reagents are mixed until homogeneous
- Do not use formamide or heat denaturing on samples containing Sequencing reagents
- Volumes less than 10 µl, add water to bring volumes to 10 µl before adding Sequencing reagents
- For 96-well reactions with volumes less than 20 µl, do not add water to bring volumes to 20 µl before adding Sequencing reagents
- If particles are visible in the Activator solution, heat the solution to 37°C and mix to re-dissolve. Cool to room temperature before using.

b) Purification Protocol and Cycle Sequencing Reaction

Based on your plate and reaction size, calculate the volume of *Sequencing Purification (SAM Solution)* and *Sequencing Purification (BDX)* required. The volumes below include an additional 10% to account for dead volume and pipetting loss.

Table 6: For 24 samples + 1 dead volume, 20µl reactions

Reagent	Volume / Well (µl)	Volume / Plate (µl)	Nb of reactions
Sequencing Purification (SAM Solution)	90 µl	4500 µl	24
Sequencing Purification (BDX)	20 µl	1000 µl	24

Table 7: For 48 samples + 1 dead volume, 20µl reactions

Reagent	Volume / Well (µl)	Volume / Plate (µl)	Nb of reactions
Sequencing Purification (SAM Solution)	90 µl	9000 µl	48
Sequencing Purification (BDX)	20 µl	2000 µl	48

1. Vortex the *Sequencing Purification (BDX)* container at maximum speed for at least 10 seconds, or until it is homogeneous.
2. Using a wide-bore pipette tip, add the calculated volume of *Sequencing Purification (BDX)* to a clean container.
3. Using a conventional pipette tip, add the calculated volume of *Sequencing Purification (SAM Solution)* to the same container.
4. Mix the reagents until homogeneous (This premix can be stored at 4°C for up to 5 days. Make sure to mix well before use.)
5. Add the premix to each well; volumes are stated below:

Table 8: Premix volume per well

Reaction volume per well	Volume of premix / Well
96-well, 20 µl	110.0 µl

6. Seal the reaction plates using heat seal or adhesive film. Verify that each well is sealed.
8. Vortex the reaction plate for **30 minutes** using the following conditions:

Table 9: Vortexing conditions according to instrument used

Vortexer	Speed
Eppendorf MixMate	2600 rmp
IKA MS3 Digital	2000 rmp
IKA Vortex 3	Setting 5
Digital Vortex Genie 2	2000 rmp
Taitec MicroMixer E-36	Maximum

9. Spin the plate at 1000 x g for at least one minute in a swing-bucket centrifuge.
10. When using the BigDye XTerminator run module, remove the seal from the reaction plate and place it in the capillary electrophoresis instrument. When using normal run modules, transfer 20µl of supernatant to a clean plate and place it in the instrument.

To avoid handling errors, identify properly the Sequencing Reactions tubes for further downstream sequencing.

6. Capillary electrophoresis

The samples are now ready for downstream capillary electrophoresis. You can place the reaction plate in the DNA analyzer and follow the instructions for use of the capillary electrophoresis instrument.

The purified extension products can be analyzed by capillary electrophoresis on one of the following platforms:

- Applied Biosystems 310 DNA sequencer
- Applied Biosystems / Hitachi 3100 (Avant) Genetic Analyzer
- Applied Biosystems / Hitachi 3130 (XL) Genetic Analyzer
- Applied Biosystems / Hitachi 3500 (XL) Genetic Analyzer
- Applied Biosystems / Hitachi 3730 (XL) DNA Analyzer
- Applied Biosystems SeqStudio Genetic Analyzer System
- Promega Spectrum Compact CE system

Note:

- The operator shall select the correct tray and retainer assemblies for the tubes and downstream DNA analyzer.
- The user shall select the correct reagents and consumables and operate as specified in the manufacturer instructions for use for the whole process of Sanger sequencing for the selected DNA analyzer.
- The user shall use a calibrated DNA analyzer and related software for the sample quality control.
- This assay is optimized to run with Filterset Z for BigDye Terminator v3.1 chemistry. Refer to your instrument manual on how to calibrate with this Dye Set.

7. Specimen quality control

There is no direct sample quality control between the amplification steps and post sequencing. The only way to quality control the samples is the review of the quality report output from the DNA analyzer software.

For primary base calling, the easiest option is to use Sequencing Analysis Software, provided with the automated sequencer. We recommend using the KB Base Caller, in combination with a DyeSet/Primer file, suitable for

BigDye v3.1 chemistry. For improved basecalling with longer read lengths, we recommend PeakTrace (https://www.nucleics.com/peaktrace/).

8. Limitations

- ***DeepChek® SANGER SEQUENCING REACTION (RUO)*** is for Research Use Only (RUO). Not for use in diagnostic procedures.
- The kit is to be used by personnel specially instructed and trained in RT-PCR procedures and sequencing.
- Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to the next, users perform method correlation studies in their laboratory to qualify technology differences. Perfect concordance between the results should not be expected due to differences between technologies. Users should follow their own specific policies/procedures.
- Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

9. Warnings and Laboratory precautions

- DNase contamination which might cause degradation of the template DNA
- DNA or PCR carryover contamination resulting in false positive signal

We therefore recommend the following:

- **Frequent cleaning of the wells of the Thermal Cycler plate.**
- Any specimen involved in this test shall be regarded as infectious substance and using good laboratory procedures as outlined in the CLSI Document M29-A4[1].
- To make sure an accurate and reliable result, always use DNase/RNase-free disposable pipette tips, tubes, and calibration pipettes.
- Use separated and segregated working areas: 1) Reagent preparation area – preparing the reagents for amplification, 2) sample preparation area- isolation of the RNA/ DNA from sample and control, and 3) Amplification area- amplification and detection of nucleic acid target.
- To avoid contamination, all the objects should be used in certain areas. All apparatus must be cleaned after each experiment.
- To avoid the contamination of fluorescent materials, disposable gloves, tubes, pipette and filter tips should not contain fluorescent material.
- Nucleic acid samples stored at -70 ° C should be thawed, mixed, and centrifuged at low temperature for a short time before use.
- Try to avoid the generation of air bubbles when the reaction solution is dispensed.
- When adding the sample, the sample should be completely added to the reaction solution, and no sample should adhere to the tube wall. The tube cap should be closed as soon as possible after the sample is added.
- The reaction tube containing the reaction solution should be capped or packed in a sealed bag and then transferred to the sample processing area.
- Check whether the reaction tubes are tightly closed before loading on the machine to avoid the leakage contaminating the instrument.
- After the amplification, the reaction tube was taken out, sealed in a special plastic bag, and discarded at the designated place.

[1] Protection of Laboratory Workers From Occupationally Acquired Infections, 4th Edition (M29-A24)(CLSI, M29A4E), https://clsi.org/standards/products/microbiology/documents/m29/

- The used tips should be thrown into disposal bottle which have 10% sodium hypochlorite solution and discarded with other waste.
- Use 10% sodium hypochlorite, 75% alcohol and ultraviolet light to disinfect the workbench and experimental items regularly.
- The Thermal Cycler instrument requires frequent calibration.
- Material Safety Data Sheets are available upon request.

10. Product quality control

In accordance with ABL's Quality Management System, each lot of the assay is tested against predetermined specifications to ensure consistent product quality. Certificates of Analysis are available upon request.

11. Symbols

Σ <N>	Contains reagents enough for <N> reactions		Consult instructions for use
	Caution		Temperature limitation
REF	Catalog number	SN	Serial Number
	Use by	**R*n***	R is for revision of the Instructions for Use (IFU) and n is the revision number
	Manufacturer		Distributor
CC	Country and date of manufacturing		

12. Contact Information

For technical assistance and more information, please see our Technical Support Center at Online: https://support-diag.ablsa.com; Email: support-diag@ablsa.com; Call +339 7017 0300 Or contact your ABL Field-Application Specialist or your local distributor. For up-to-date licensing information or product-specific disclaimers, see the respective ABL Assay User Guide. ABL User Guides are available at **www.ablsa.com/ifu** or can be requested from ABL Technical Services or your local distributor.

13. Manufacturer and distributors

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