Fragment Analyzer™ CRISPR Discovery Gel Kit

INSTRUMENT

Manufacturer: Advanced Analytical Technologies
Distributor: Kem-En-Tec Nordic (info@kem-en-tec.dk)
Technical support: Klaus T. Nielsen (kn@kem-en-tec.com)

Owner: University of Tampere / LAS
Location: ARVO E215
Responsible person(s): Hanna Selin , tel. 040 1901 260, email: hanna.selin@uta.fi
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RESERVATIONS *(billing according to reservations)* in AGENDO:
https://next.cirklo.org/biomeditech/

ANALYSIS KIT:

CRISPR Discovery Gel Kit, #DNF-970

- Sizing range: 1 – 6000 bp
- Input concentration range: 0.005 – 2 ng/µl

REAGENTS:

Stored at +4 °C Arvo E218 cold room	**Stored at -20°C** Arvo E217 freezer room	**Stored at RT** Arvo E215 (at FA)
dsDNA 810 Separation Gel	Intercalating Dye *(same for all Fragment Analyzer Analysis Kits)*	5x Capillary Conditioning Solution
1x & 5x dsDNA Inlet Buffer		
1x dilution of Capillary Conditioning Solution	Mutation Detection DNA Ladder	Mineral oil
Dilution Buffer E 0.1X	1 bp 6,000 bp Markers	Gel/Dye Mixture (at FA)

- Reagents are stored in room E215 (RT), E217 freezer room or E218 cold room. Opened reagents and dilutions *(1x Capillary Cond. & 1x Inlet buffer)* are marked with opening date and/or ”X”.
- Please open a new reagent tube or bottle in order of the expiration dates and mark the opening date and/or “X” to the tubes
- Take the reagents to warm up to room temperature (RT) for 30 minutes prior to use
- All reagents are harmless, they can be discarded by pouring down the sink

OTHER SUPPLIES: *(stored at the shelf or drawer under the instrument table or above the sink)*

- Capillary Storage Solution *(AATI #GP-440-0100-100/Kem-En-Tech)*
- Specified 96 Well Semi-Skirted PCR Plate (list of approved plates at Appendix A) for samples (drawers 1-3) and Rinse buffer (drawer M)
- 96-well DeepWell 1 mL Plate *(Fisherbrand #12-566-120/Kem-En-Tech)* (drawer W)
- BD Falcon 50 ml centrifuge tubes *(BD #352070, #734-0448/VWR)*
- Pipettes (Biohit mLine and 12-channel Thermo ClipTip)
- Electronic dispenser (Biohit) an dispenser tips (0.5/1.0/2.5 ml)
- Serum pipettes *(for discarding 1x Inlet Buffer from the 96 DeepWell 1 mL Plate in row A)* and serological 10 ml pipettes
- Microplate seals *(#4ti-0510/Biotop)*

QUICK CHECK LIST *(for more instructions, see the specific sections)*

1. Take the reagents to RT at least 30 minutes prior to performing the run

   From the cold room:
   - 1x (or 5x) Inlet Buffer
   - dsDNA 810 Separation Gel (in case of new gel/dye mixture is required)
   - 1x Capillary Conditioning Solution
   - Dilution Buffer E 0.1X

   From the freezer room:
   - Aliquoted Mutation Detection DNA Ladder
   - 1 bp and 6,000 bp Markers
   - Intercalating dye (in case of new gel/dye mixture is required)

2. Turn on the computer and the instrument
3. Turn on the ***Fragment Analyzer Software*** (User ID: *"Administrator"*, no password).
4. Check the **Waste Bottle** and the **Waste plate** at the Waste drawer (W) and empty if needed. Update the solution level to the software *(Utilities – Solution levels).*
5. If necessary, insert (or add) the **Gel/Dye mix** solution to the desired Gel fluid line (1 or 2) and update the solution level to the software *(Utilities – Solution levels).*
6. Check the level of **Capillary Conditioning Solution** and add or replace if necessary and update the solution level to the software *(Utilities – Solution levels).*
7. Replace the **1x Inlet Buffer** in the 96-well Midi plate at row A in the Buffer drawer (B) (once a day). Press "*Park*" – replace the buffer – place the plate back – press "*Buffer*"
8. Place **1 bp and 6,000bp** Marker solution into Drawer M.
9. Prepare the sample plate and load into one of the three sample trays. If you place a plastic seal on the plate in order to spin the plate, **remember to remove the seal before running the plate to avoid capillary damage!**

NOTE! When using the instrument for the first time, contact the person(s) responsible for introduction!

PROTOCOL

1. GEL/DYE MIX PREPARATION

- A gel/dye mix is valid for up to 2 weeks after preparation.

- If necessary, prepare a fresh mixture:

1. Bring the **Separation Gel** and **Intercalating Dye** *(same for all kits)* to RT prior to mixing.

2. Add an appropriate volume of Intercalating Dye to Separation Gel in a 50 mL tube according to the table below.
   - ***NOTE! When switching applications between RNA/NGS/gDNA/CRISPR kits at the certain Gel fluid line 1 or 2 you need to do 5 mL more of the mix due to priming*** *– see the example for 12 samples at the table and the step 7.*

# of samples	Volume of Intercalating Dye	Volume of Separation Gel
12	1,0 µl *(1,5 µl for priming)*	10 mL *(15 mL for priming)*
24	1,5 µl	15 mL
36	2,0 µl	20 mL
48	2,5 µl	25 mL
60	3,0 µl	30 mL
72	3,5 µl	35 mL
84	4,0 µl	40 mL
96	4,5 µl	45 ml

3. Mix by gently inverting the tube – do not vortex!

4. Mark the date on the tube.
   - NOTE! If there is still less than 2 weeks old mixture left at the instrument, a fresh gel/dye mixture can be mixed with the old one to achieve the required total amount of the mixture *(e.g. if there's still 5 mL of the mixture left, add 0,5 µl of Intercalacting Dye and 5 mL Separation Gel to obtain required 10 mL for 12 samples)*. Do not change the date

5. Place the gel/dye mixture onto the instrument and insert into the desired ***Gel fluid line*** (1 or 2). Ensure the fluid line is positioned at the bottom of the conical tube.

6. Update the solution levels in the Fragment Analyzer instrument control software. From the ***Main Menu***, select ***Utilities – Solution Levels***. A menu will be displayed to enter in the updated fluid levels

7. **PRIMING:** When switching applications between RNA/NGS/gDNA/CRISPR kits on the certain Gel fluid line 1 or 2, prime the fluid line after loading fresh gel/dye mix. From the ***Main Menu*** select ***Utilities – Prime*** – select the desired fluid line 1 or 2.

8. Leave the gel/dye mixture in the instrument after the run.

2. CAPILLARY CONDITIONING (CC) SOLUTION PREPARATION

- Capillary Conditioning Solution is the same for all kits.
- Place the 1x CC Solution onto the instrument in the Conditioning Solution location in a 50 mL Falcon tube *(a typical 12-capillary experiment cycle consumes less than 4 mL but the instrument requires at least 10 mL in the tube)*.
- Update the solution levels in the Fragment Analyzer instrument control software. From the ***Main Menu***, select ***Utilities – Solution Levels***. A menu will be displayed to enter in the updated fluid levels.
- 1x CC Solution should be added to the system as use demands or if it hasn't been changed within 2 weeks.
    - NOTE! If there is less than 2 weeks old 1x CC Solution left at the instrument, new 1x CC Solution can be mixed into the same tube to achieve the required total amount *(e.g. if there's still 5 mL of the 1x CC solution left, add 5 mL of new 1x CC solution to obtain required 10 mL for 12 samples)*. Do not change the date on the tube!
- If necessary, prepare the **1x** Capillary Conditioning Solution to a 250 mL glass bottle by adding 50 mL of the **5x** Capillary Conditioning Solution to 200 mL of Milli-Q water. Mix by gently inverting the bottle. Mark the date and your initials. 1x CC Solution can be used up to **2 weeks** after preparation when stored in the instrument or **3 months** when stored at +4 °C. Store most of the 1x buffer at +4 °C and leave an appropriate aliquot in the instrument.

3. INLET BUFFER PREPARATION

- Inlet Buffer is the same for all kits. Bring the **1x or 5x Inlet Buffer** to RT prior mixing and use.
- 1x Inlet Buffer in the 96-well Deep Well Plate in the Buffer drawer (B) **has to be replaced daily:**
    1. From the ***Main Menu*** press "***Park***"-icon to bring the plate to the tray B.
    2. Remove the old buffer from the **row A** by pipetting it to the sink with a serum pipette. Add exactly 1 ml of 1x Inlet Buffer to every well at row A in the 96 DeepWell Plate. Do not overfill!
        - **Note!** *The Storage Solution is at the same plate at **row H** and is replaced only once in a month, so empty only the row A!*
    3. Place the plate back to the tray B and press ***"Buffer"***.
- If necessary, prepare the **1x Inlet Buffer** to a 250 mL glass bottle by adding 50 mL of the **5x Inlet Buffer** to 200 mL of Milli-Q water. Mix by gently inverting the bottle. Bring the 5x Inlet Buffer to RT prior diluting. Mark the date and your initials. 1x buffer can be used up to **3 months** after preparation when stored at +4 °C.
- **Replace the 96-well Deep Well Plate and Capillary Storage solution monthly.** When taking a new Deep Well Plate, pipette exactly 1.1 ml of Capillary Storage Solution to the wells at **row H**. Mark the date to the plate. Row H is used for the store location and the array moves to this position at the end of the experimental sequence.

4. INSTRUMENT PREPARATION (waste containers and rinse buffer plate)

- Check the fluid level of the waste bottle and waste tray (drawer W) and empty as needed. Update the waste bottle solution level to the software.

- Prepare a new 96-well plate filled with **30 µl/well** of **1 bp and 6,000 bp Marker** solution (ready-to-use solution in 1X TE buffer) at **row A** using the electronic dispenser pipette daily. Mark the date on the plate. Agitate and centrifuge the vial before dispensing. If necessary, spin the plate shortly to reduce air bubbles. Cover the wells with 20ul/well of the supplied mineral oil to allow reuse for at least 30+ injections. After the run, cover the plate with a seal tape and store at +4C (can be reused for at least month).

- Use only specified semi-skirted 96-well PCR plates (Appendix A) in the instrument. Using wrong kind of plates may lead to capillary damage!

- Place the prepared **Marker plate** into drawer "**M**". Ensure the plate is loaded with well A1 towards the back left on the tray.

5. SAMPLE PLATE PREPARATION

- Allow the **Mutation Detection DNA Ladder** to warm up to RT prior to use. It is a ready-to-use solution in 0.1X TE buffer.

- Use only specified semi-skirted 96-well PCR plates (Appendix A) in the instrument. Using wrong kind of plates may lead to capillary damage!

- Run the samples immediately once prepared or cover the plate with a cover film, store the plate at +4 °C and run ASAP. Alternatively, to prevent evaporation, place a mineral oil overlay on each sample (50 µl/well).

  1. Pipette **22 µl of the Dilution Buffer E 0.1X** to each well in a row that is to contain **sample** with an electronic dispenser pipette.
  2. Fill any unused wells within the row with **24 µl/well of Dilution Buffer E 0.1X**.
  3. Pipette **24 µl of Ladder** into the well 12 in every row to be analyzed.
  4. Pipette **2 µl of each sample** into the wells 1-11 containing 22 µl of Dilution Buffer E.
  5. **Mix well** by either swirling the pipette tip while pipetting up/down or using the 12-channel pipette set to 20 µl for mixing.
  6. Check that there are no air bubbles trapped in the bottom of the wells. If necessary, remove the bubbles by either centrifuging the plate shortly in a mini plate spinner, tapping it against the table or using a clean pipette tip.
  7. Place the Sample plate in one of the three sample trays (drawers 1, 2 and 3). Load (or create) the experimental method.

6. PERFORMING AN EXPERIMENT

1. To set up an experiment, from the Main Menu of the Fragment Analyzer instrument control software, select the **Operation** tab.

2. Select the sample tray location (1, 2 or 3) by left clicking the **Sample Tray #** dropdown or by clicking the appropriate **sample plate tab** and choosing the appropriate location.

3. Left click a well of the desired sample plate row. Enter the sample name if desired into the respective **Sample ID** cell by left clicking the cell and typing the name or import the sample information from .txt or .csv file by selecting the **Load from File…** -option.

4. Under the **Run Selected Group** field press **Add to queue**. The **Separation Setup** pop-up form will appear to the screen:
   - From the **Method** dropdown menu select the right method:
     - ***CRP-910-33 –CRISPR Discovery.mthds***
   - Select the **Gel** line (1 or 2)
   - The **Tray Name** can be entered if it's necessary to identify the sample plate.
   - The **Folder Prefix** can be entered to amend the folder name for the results
   - The results can be copied to another directory location by checking the **Copy Results** box and selecting the desired **Copy Path**
   - Any **Notes** can be entered regarding the experiment

5. Once all information has been entered, press **OK** to add the method to queue. After a row (or tray) has been added to the queue, the method(s) will be listed on the main screen under the **Method Queue**. Repeat the steps 4-5 for any remaining sample rows. Additional experiments can be programmed and added to the Method Queue at any time while the instrument is running.

6. Once the experiment has been loaded onto the queue, the method can be viewed or edited by pressing the **Method Summary** field.

7. Prior to starting the experiment, verify all trays (buffer/storage, rinse, waste, sample, etc.) have been loaded into their respective drawer locations!

8. Press the **Play** icon to start. To clear the run queue of all loaded runs, press the **Clear** icon.

7. AFTER COMPLETION OF THE EXPERIMENT(S)

- After completion of the last queued experiment, the instrument stage will automatically move to the Store location.

- **Throw away the Sample plate** and **empty and rinse the waste tray** (from drawer W). **Seal and store the Marker plate** at +4C. Update the waste solution level in the Fragment Analyzer instrument control software. From the *Main Menu*, select *Utilities – Solution Levels*. A menu will be displayed to enter in the updated fluid levels.

- **Turn off the instrument and the computer.**

8. PROCESSING EXPERIMENTAL DATA

1. The data is viewed and processed using PROSize 2.0 software. You can use PROSize on Fragment Analyzer's computer or on your own computer. If you want to install the PROSize program on your own computer, ask Heini Kallio (heini.kallio@uta.fi) to give you the PROSize installer.

2. Open your run by clicking File – Open File. Select the correct file and click 'Open'.

3. **Look at the markers and ladder first**. The data is normalized to the lower and upper marker and calibrated to the Mutation Detection DNA Ladder. In principle, as long as the ladder looks good, the results are reliable (a total of 16 peaks should be observed, refer to the user guide to see the representative ladder results). If the ladder is not ok, you can import ladder from a previous successful run. Select Analysis – Show size calibration. Choose 'Export' and save the ladder to your folder. Then open the run where the ladder was not optimal. Select Analysis – Show size calibration. Select 'Use imported ladder profile' and choose the saved ladder from your folder.

4. **Remember to change the dilution factor if your dilution is not 12** (2 µl sample + 22 µl Dilution Buffer E 0.1X). It can be changed by clicking a tool icon on the upper right corner of a peak table that says 'Set individual parameters'. Select 'Quantification', change the dilution factor and press 'Apply to selected' or 'Apply to all'.

5. **If the software hasn't placed the lower and upper marker correctly for a certain sample, you can manually correct this**. Select the individual sample that has the incorrect peak(s) called as markers and right-click on top of the correct peak(s). Choose 'Set as lower marker' or 'Set as upper marker'.

6. For **CRISPR samples**, a positive assay result is indicated by the presence of cut DNA fragments whose size sum to the intact, priginal DNA fragment within reasonable sizing error %.

    - Using a separate **CRISPR plugin for Prosize 2.0**, you can manually enter the expected PCR fragment size as well as the expected cleavage fragment sizes, and automatically score and highlight detected cut fragments.
        - In Prosize 2.0, open the file you wish to analyze.
        - For the main menu, select **Tools – CRISPR** and open the file "**CRISPR Main**".
        - To **configure the fragment sizes** for the Plugin, select Configure and enter the expected PCR fragment sizes for each sample well. By right-clicking you can copy entered values either to columns/rows/selection. Fragment configurations can be saved and loaded.
        - The **% Error field** determines what +/- percentage sizing error is allowed between the expected and measured size (default 5 %).
        - The **Max cleavage %** determines the treshold below which the % Cleavage value is no longer highlighted in the main CRISPR plugin screen, to avoid false positives of 100 %. A recommended value for this setting is between 50-70 %.
        - Once configuration is set, **exit** to the main screen.
    - The Plugin will **automatically color code the peaks** that correspond to the input sizes if they fall within the % Error range. The Plugin will **automatically calculate** the molar concentration of each peak, as well as the **% Cleavage value**.
    - Results can be output as PDF or .csv file.
    - For troubleshooting, refere to the manufacturer's User Manual.

7. **Exit the program by selecting File – Exit. There is no need to save your changes. The changes are automatically saved when you exit the program.** In case you want to return to the original raw data without any changes made by you, go to drive C – AATI – Data and delete .ANAI and .PKS files from your run's folder. This will delete your changes and you can start analyzing the raw data from the beginning.

8. **In case of technical problems, contact Klaus T. Nielsen** (kn@kem-en-tec.com). It is advisable to zip your results and send them to Klaus; Select Help – Zip opened data file.

Appendix A

List of approved Sample/Marker PCR Plates (semi-skirted)

Approved Vendor/Part Number; Description

- VWR # 83007-374; VWR® 96-Well PCR Plates, Half-Skirted Plates, Natural
- VWR # 89049-178; VWR® 96-Well Thermal Cycling Plate
- Eppendorf # 951020303 (various colors); Eppendorf* 96-Well twin.tec* PCR Plates, Semiskirted
- MidSci Pryme # AVRT1; Pryme PCR Ergonomic Plates, 96x0.2ml, Semi-Skirted, Natural
- BioRad Hard-Shell # HSS-9601; Hard-Shell® Full-Height 96-Well Semi-Skirted PCR Plates
- Greiner Bio-One # 652280; 96W PCR Microplate, Polypropylene, Half-Skirt, Natural, No Lid
- 4titude Framestar # 4ti-0900, -0950, -0770/C; FrameStar® 96 semi-skirted
- Scientific Specialty # 3450-00; 96-Well "Semi Skirt" UltraFlux® PCR Plate
- Neptune # 3742.X; Semi-Skirted 96-Well PCR Plates