**TITLE: METH030\_Miseq\_LIBRARY\_PREP**

**1 INTRODUCTION**

**1.1 PURPOSE OF SOFTWARE**

Preparing DNA samples for sequencing on the MiSeq NGS platform, using PCR, E-Gel electrophoresis, MN bead cleanup, QIAxcel measurement, QIAgility equimolar pooling and Bioanalyzer measurement.

**1.2 DEFINITIONS AND ABBREVIATIONS**

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**1.3 STATUS**

The status and management of this document are regulated in the AODocs platform, the foundation wide accessibility is provided on [N=Info](https://sites.google.com/a/naturalis.nl/social-intranet/home/support/sops).

**2 RESPONSIBILITIES & PEOPLE**

**2.1 RESPONSIBILITIES**

|  |  |
| --- | --- |
| **FUNCTION** | **ROLES and RESPONSIBILITIES** |
| Lab workers (Researchers, Students, Guests) | Is able to use this document if contributing to the DNA barcoding work process |
| (Senior) Research Technicians | Knows these document instructions and is able to instruct other lab workers who need to use the Geneious program. |
| Head Laboratories | Has final responsibility for all laboratory workflow and instructions |

**2.2 LIST OF PEOPLE ADDRESSED**

Elza Duijm: 1st responsible for NGS post lab, Ion Torrent, QIAxcel and QIAgility

Marcel Eurlings: 2nd responsible for NGS post lab, Ion Torrent, QIAxcel and QIAgility

**3 ADDITIONAL AIDS AND RESOURCES**

**4 INSTRUCTIONS**

**4.1 1st PCR DNA TEMPLATE**

DNA template has to be amplified with a 20μl PCR using Nextera-tailed primers. A PCR sheet template, containing reaction mixes, primers and PCR programs is available in

B:\DNA Barcoding\Templates\PCR\_template\_NXT-1\_20ul\_v1.4

**Do not use more then 35 cycles for this first round PCR!**

Sequences Nextera tails:

Forward: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'

Reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'

**4.2 E-Gel**

PCR products have to be checked on an E-Gel, using working instruction [WI\_SP0004\_E-GEL](https://docs.google.com/document/d/1LqNABXWoamNklrqD-fM0HJ68H11K1TI2JDbCbkpIqIA/edit)

**Make sure you use filter tips for pipetting from your PCR sample.**

**4.3 MN BEADS CLEANUP OF NXT PCR-1**

PCR products of NXT PCR1 have to be cleaned before they can be labeled with sample-unique lables in NXT PCR2.

**4.3.1 MN beads cleanup of 96-well plate, using magnetic extractor stamp**

- Fill a 96- well round bottom plate with appropriate amount of magnetic beads

(in case of a standard clean-up 0.9 times the PCR volume)

- Add the PCR product to the magnetic beads, mix and incubate 5 minutes

- Fill 2 fresh round bottom plates with 50µl 80% EtOH (wash plates)

- Fill 1 fresh round bottom plate with appropriate (usually the volume of PCR product) amount of MQ (elution plate)

- Load the magnetic stamp with a cover plate and make sure the plate is locked into place

- Place the magnetic stamp in the sample / beads plate and wait for 1 minute

- Bring the stamp to the first wash plate and leave for 30 seconds

- Bring the stamp to the second wash plate and leave for 30 seconds

- Take out the stamp from the wash plate and let the beads dry for 30 seconds by holding it in the air

- Place the stamp in the elution plate and release the cover plate in a very quick snapping motion to prevent beads from following the magnetic pins up the sides of the cover plate.

- Swirl the cover plate in the MilliQ to release all beads, then put the cover plate back on the stamp in the same position

- Mix the beads by pipetting the solution up and down to maximize the release of DNA from the beads

- Place the stamp back into the cover plate and wait for 5 minutes

- Take out the stamp with the cover plate and discard the cover plate (with attached beads)

- Transfer the purified PCR product to a 0.2 ml PCR plate for storage

**4.3.2 MN beads cleanup of 96-well plate, using tips (optional)**

- Let the MN beads get to room temperature (remove from fridge 15 min before use)

- Prepare fresh 80% EtOH (200µL per sample, e.g. 41,7 ml EtOH 96% + 8,3 ml MQ)

- Mix the MN beads well by vortexing

- Add 0.9x (PCR product volume) MN beads to the wells of a new 96-well plate

- Add the PCR product to the MN beads and mix well by pipetting

- Incubate at room temperature for 5 minutes

- Place the plate on the magnetic rack

- When the solution is clear (5 min), carefully remove the supernatant (LEAVE THE PLATE ON THE MAGNET)

- Wash the beads 2x with 100µl 80% ethanol (LEAVE THE PLATE ON THE MAGNET)

- Let the beads air dry for 1 minute (LEAVE THE PLATE ON THE MAGNET)

- Take the plate off the magnet and resuspend the beads with e.g. 25µl MQ, by pipetting up and down

- Place the plate back on the magnet

- When the solution is clear (5 min), move 22µl of the the supernatant to a clean plate

**4.4 2nd PCR NEXTERA XT LABELS**

PCR products from the 1st PCR have to be labeled with MiSeq Nextera XT labels. 384 unique label combinations are possible. A PCR sheet template, containing reaction mixes, primers and PCR programs is available in B:\DNA Barcoding\Templates\PCR\_template\_NXT-2\_20ul\_v1.5.xlsx

Do not switch well positions within your plate (e.g. when there are empty positions) and always use a multichannel pipette to avoid confusion.

The following instructions have to be followed when preparing the Nextera XT PCR:

- Prepare master mix: clean room

- Use fresh pipette tips when adding primers from 96-well plate

- Add template: NGS post lab

**4.5 MEASURING 2nd PCR ON QIAXCEL**

**4.5.1 Sample prep**

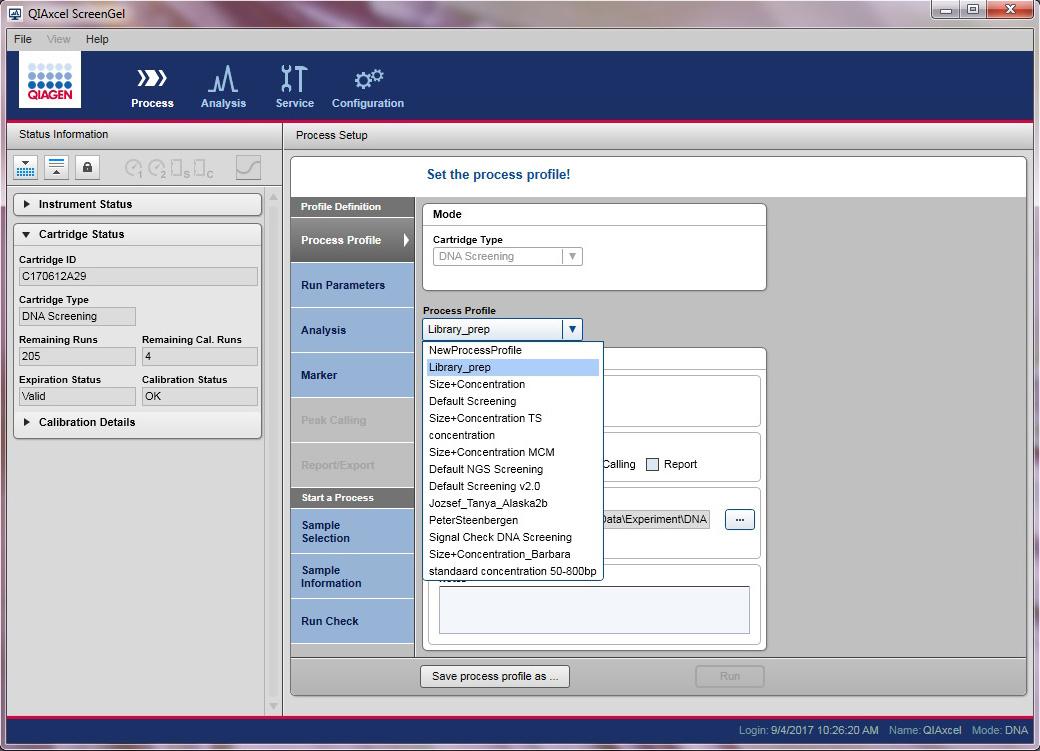
- Fill 1 well with 1:3 dilution Analysis Marker (ladder)

- Fill sample wells with 18μl MQ + 2μl 2ndPCR product

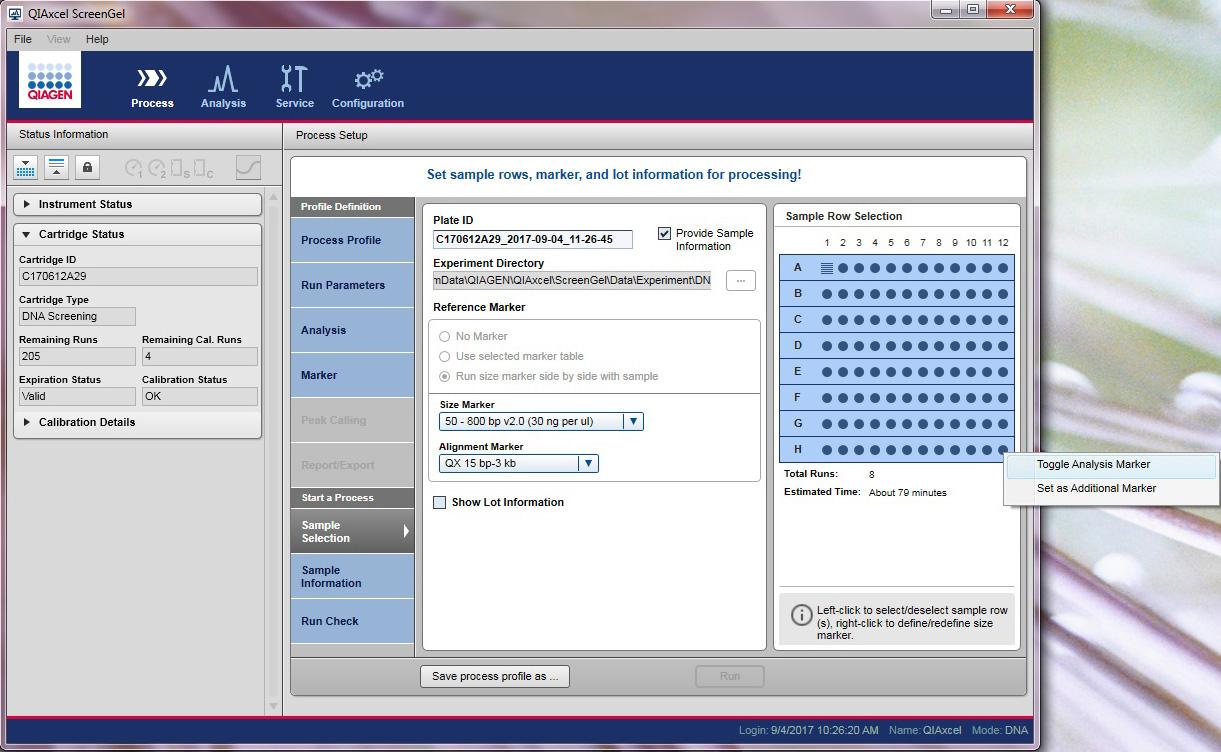
- Fill empty wells with 10μl MQ

**4.5.2 Set parameters**

- Open QIAxcel ScreenGel, user: QIAxcel pass: QIAxcel2



- \Process\Process Profile\Library\_prep



- \Sample Selection\Plate ID

- Adjust plate name

- \Sample Selection\Sample Row Selection (right window)

- Highlight relevant (all) rows by left clicking on them

- Right click on the well containing the Analysis Marker (ladder) and select \Toggle Analysis Marker

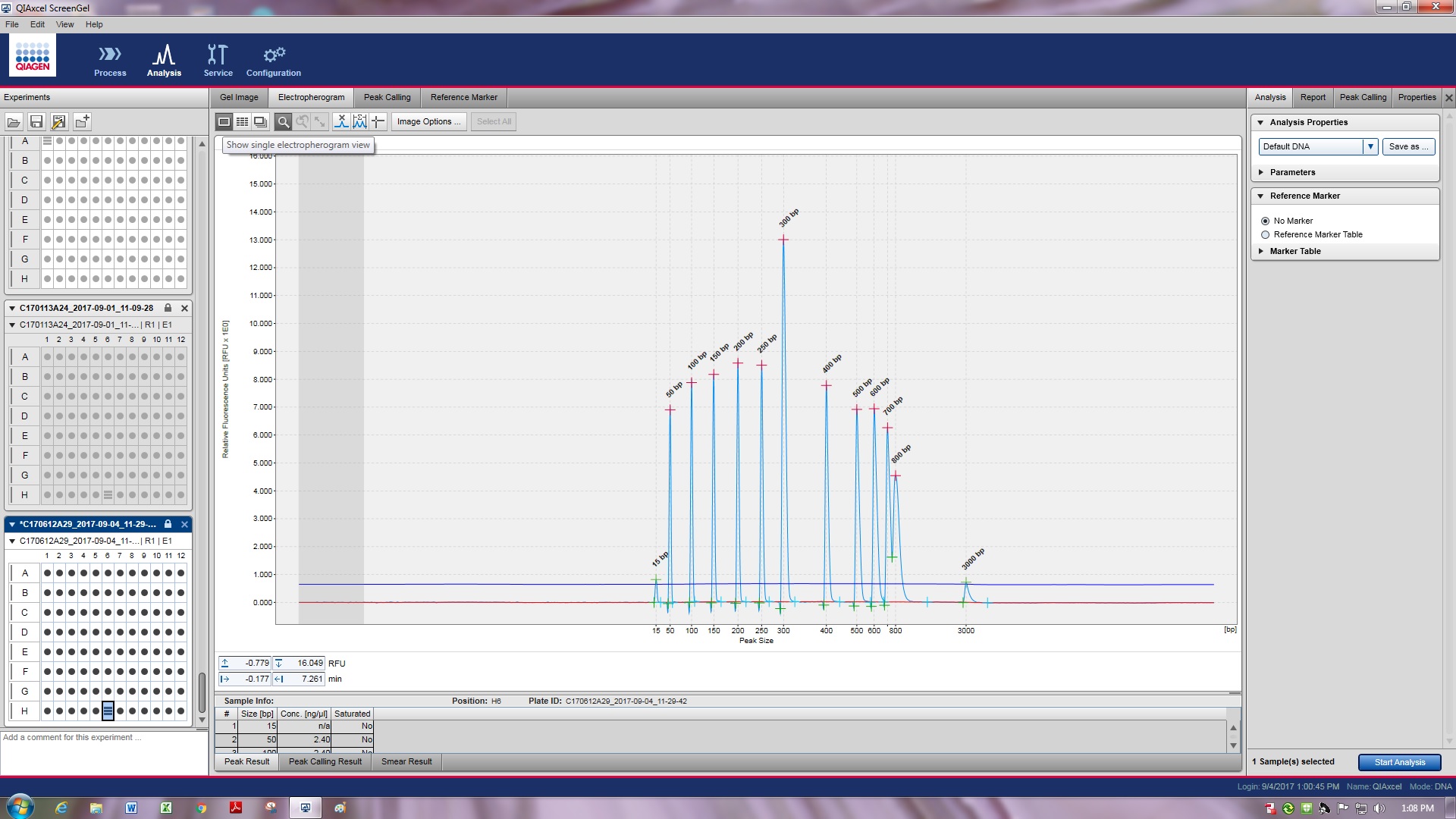
- \Run Check\Please Confirm\☑ All selected sample rows contain samples\☑ Alignment marker is loaded\☑ Size marker is loaded\Run

**4.5.3 Analysis**

- Tab \Analysis\

- After the run (80 minutes for 96 samples), select correct Plate ID, select all samples (Ctrl+A) and drag them into the right window

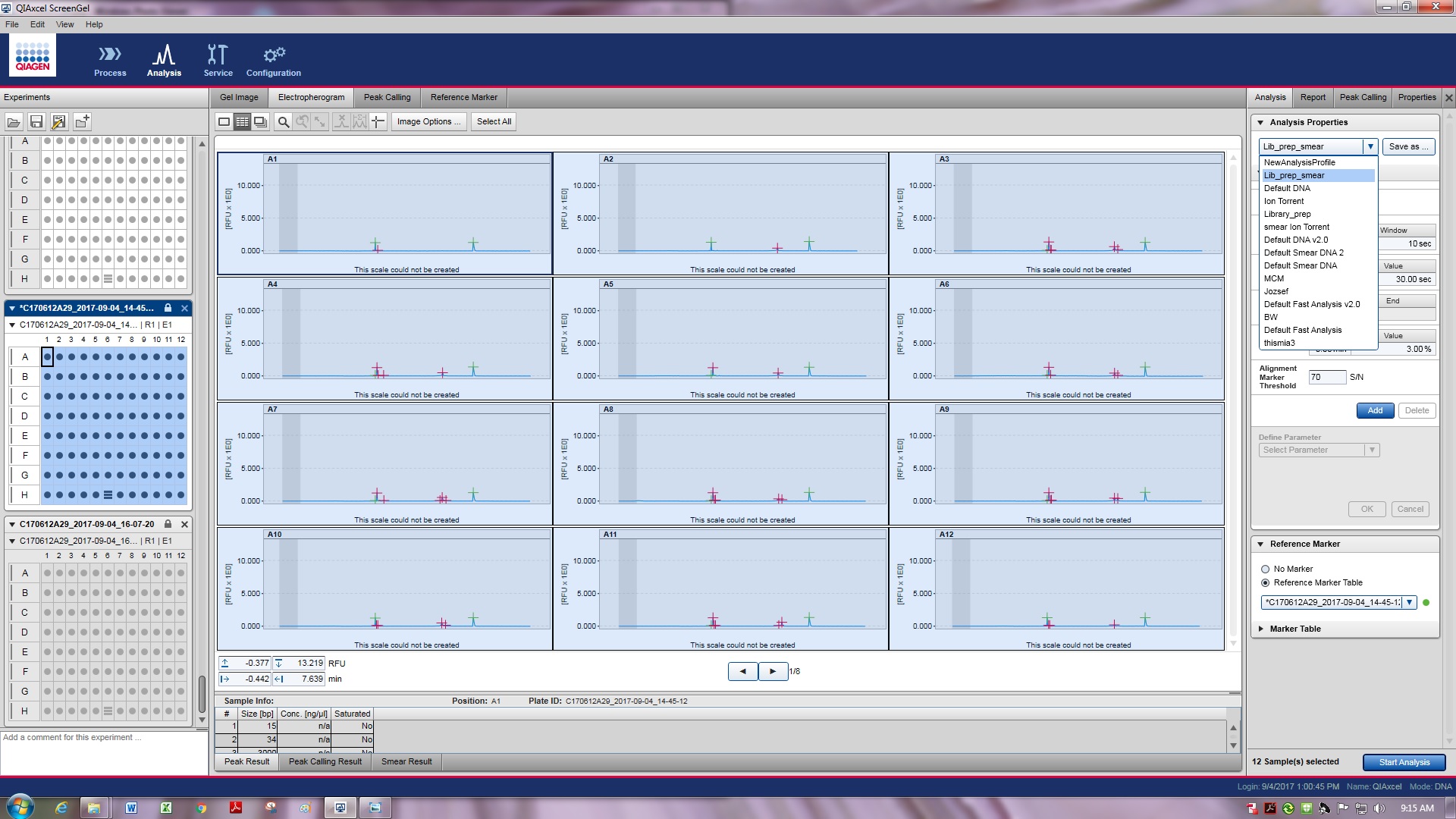
**-** Tab\Electropherogram\

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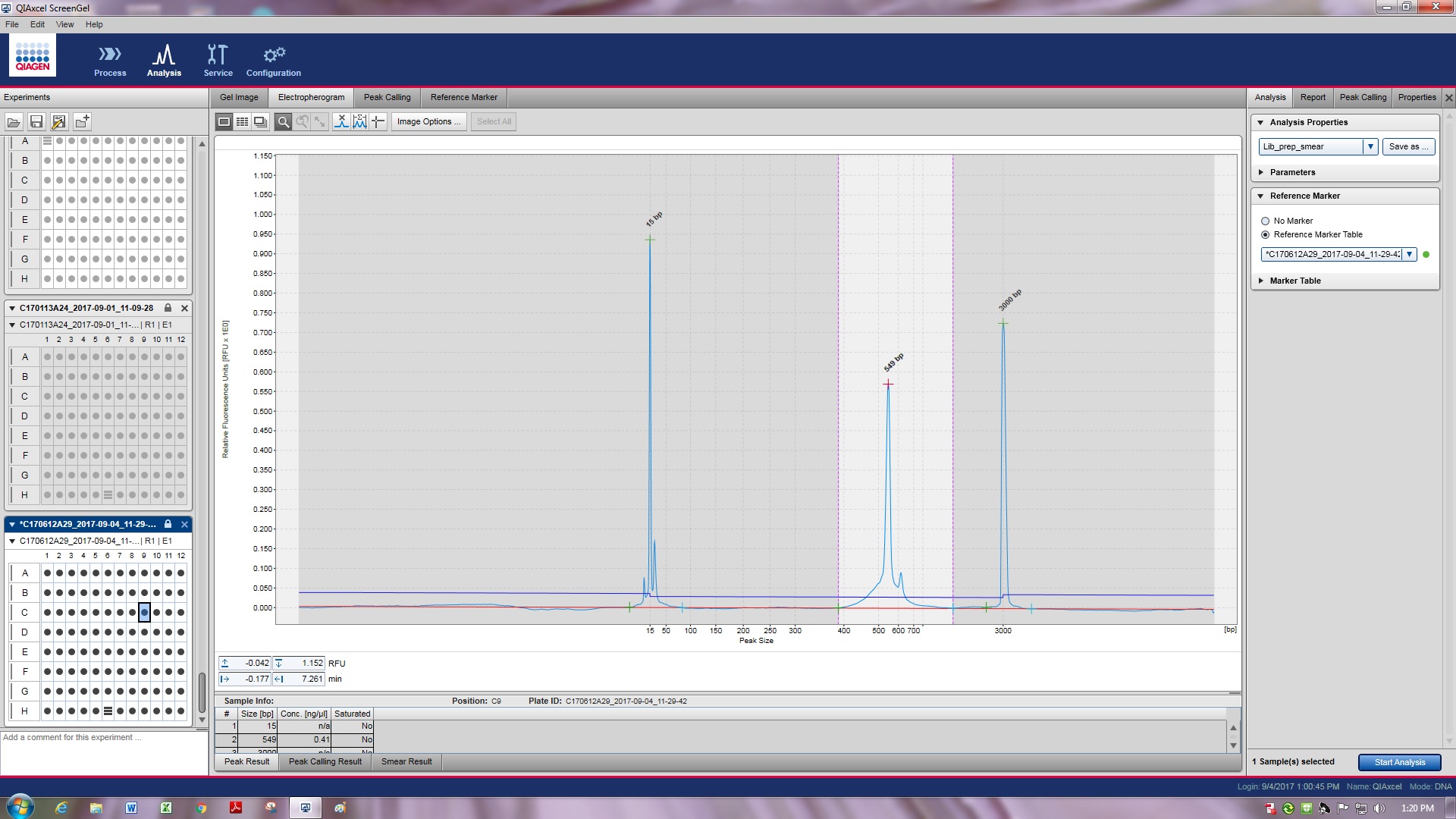
**-** Left click on the Analysis Marker and switch to \Show single electropherogram view (button top left)

- \Analysis Properties (right window) \Default DNA\Reference Marker\No Marker\Start Analysis

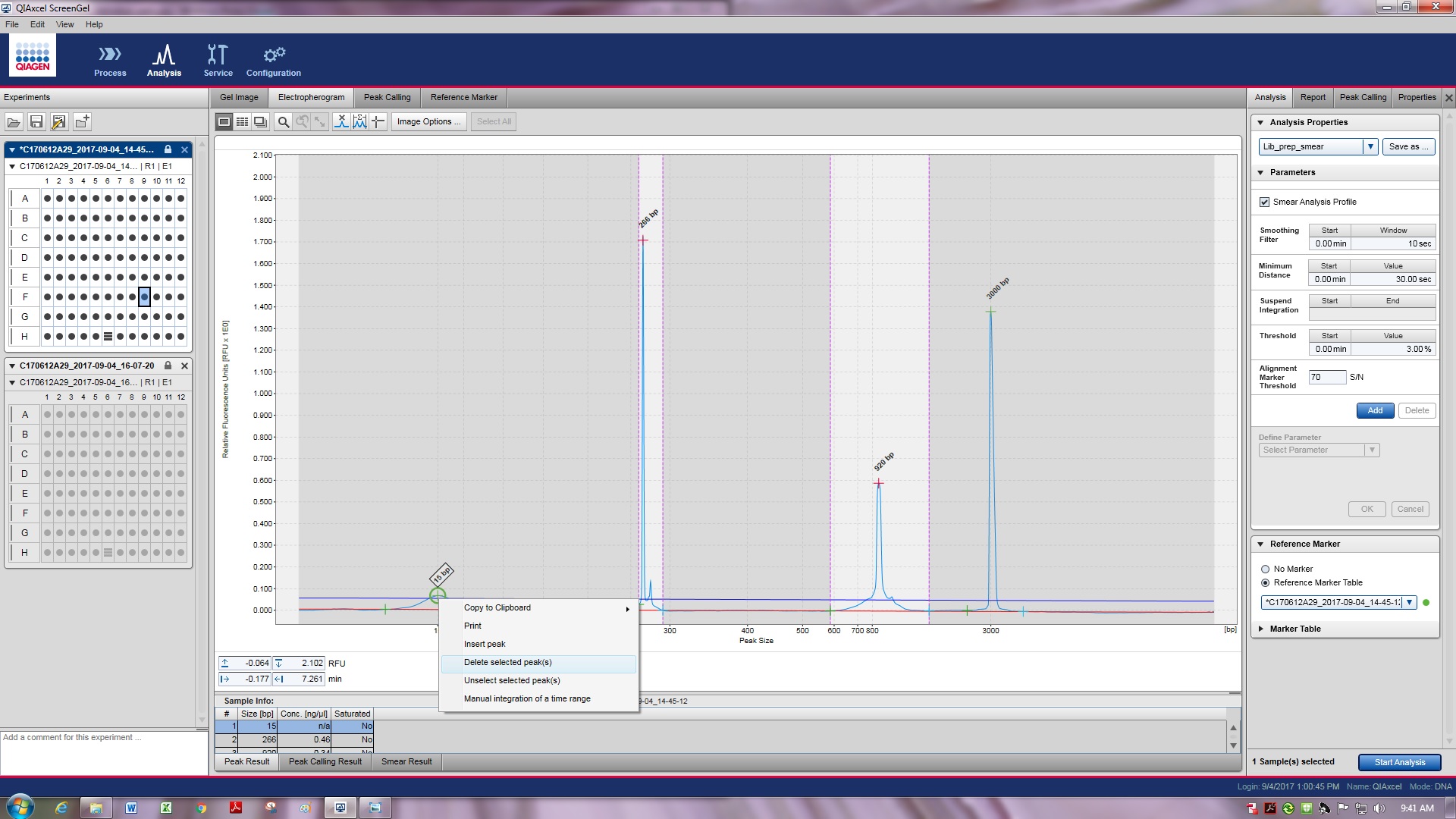
- Tab \Reference Marker\Apply



**-** Tab\Electropherogram\Select All (button top left)\Analysis Properties\Lib\_prep\_smear\Start Analysis

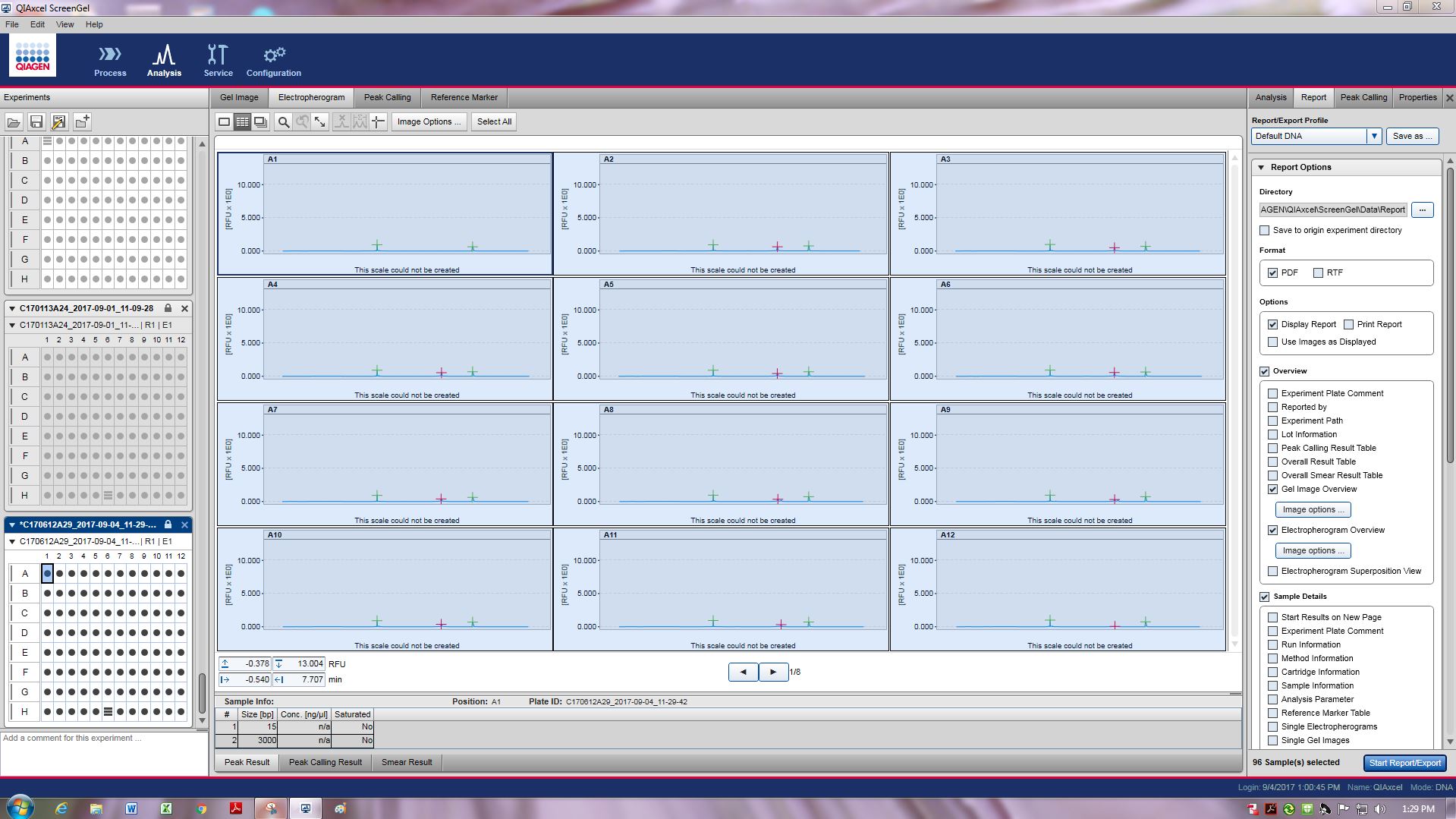


- Switch to \Show single electropherogram view and check/adjust the smear of each well



- False peaks can be removed by selecting them with left click, followed by right click \Delete selected peak(s)

**4.5.4 Report**



- \Show electropherogram overview (button top left)\Select All

- Tab \Report (right window)\☑ PDF\Start Report/Export

- Uncheck \PDF\☑ XML Export\Start Report/Export

**4.6 QIAGILITY EQUIMOLAR POOLING**

**4.6.1 Preparation QIAgility import**

- Open the XML Export e.g. C170612A29\_2017-09-04\_11-29-42\_20170904\_133105\_Ex.xml with Excel

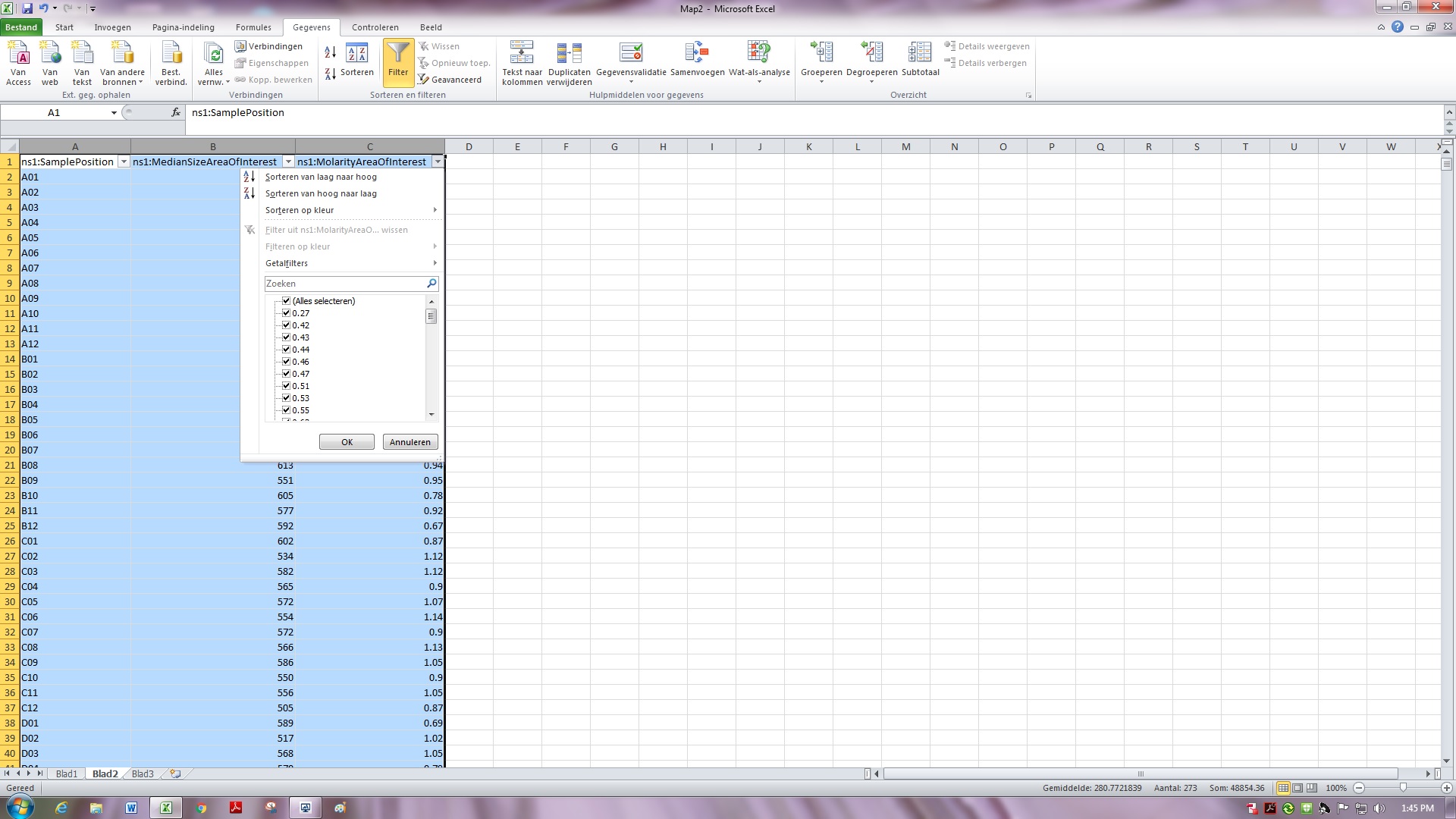
- Copy columns T (ns1:SamplePosition), AZ (ns1:MedianSizeAreaOfInterest) and BG (ns1:MolarityAreaOfInterest) to a new tab

- Select the 3 columns

- \Gegevens\Duplicaten verwijderen\Ok\Ok

- Check if there are 96 samples (97 rows) present

- \Filter



- Check if the highest value divided by the lowest value ≤ 5 \Ok

- If the above is not the case, adjust the lowest values to highest value divided by 5 (e.g. if the highest value = 2.00, adjust all values ≤ 0.4 to 0.4)

- Fill in new lowest value (in this case 0.4) in wells with no value at all

For example: ns1:MolarityAreaOfInterest

0.27 -> 0.4

0.0 -> 0.4

- \Save as\Tekst (tab is scheidingsteken) (\*.txt)\Ok\Ja

- Close Excel without saving changes to the original XML Export

**4.6.2 QIAgility import**

When the QIAgility robot cannot communicate with the PC, switch off the QIAgility, unplug and replug the QIAgility USB from the PC and restart QIAgility.

- Switch on the QIAgility liquid handling workstation before opening the QIAgility software

- Open QIAgility software

- Tab \Recent\normalize and pool\_\_\Open

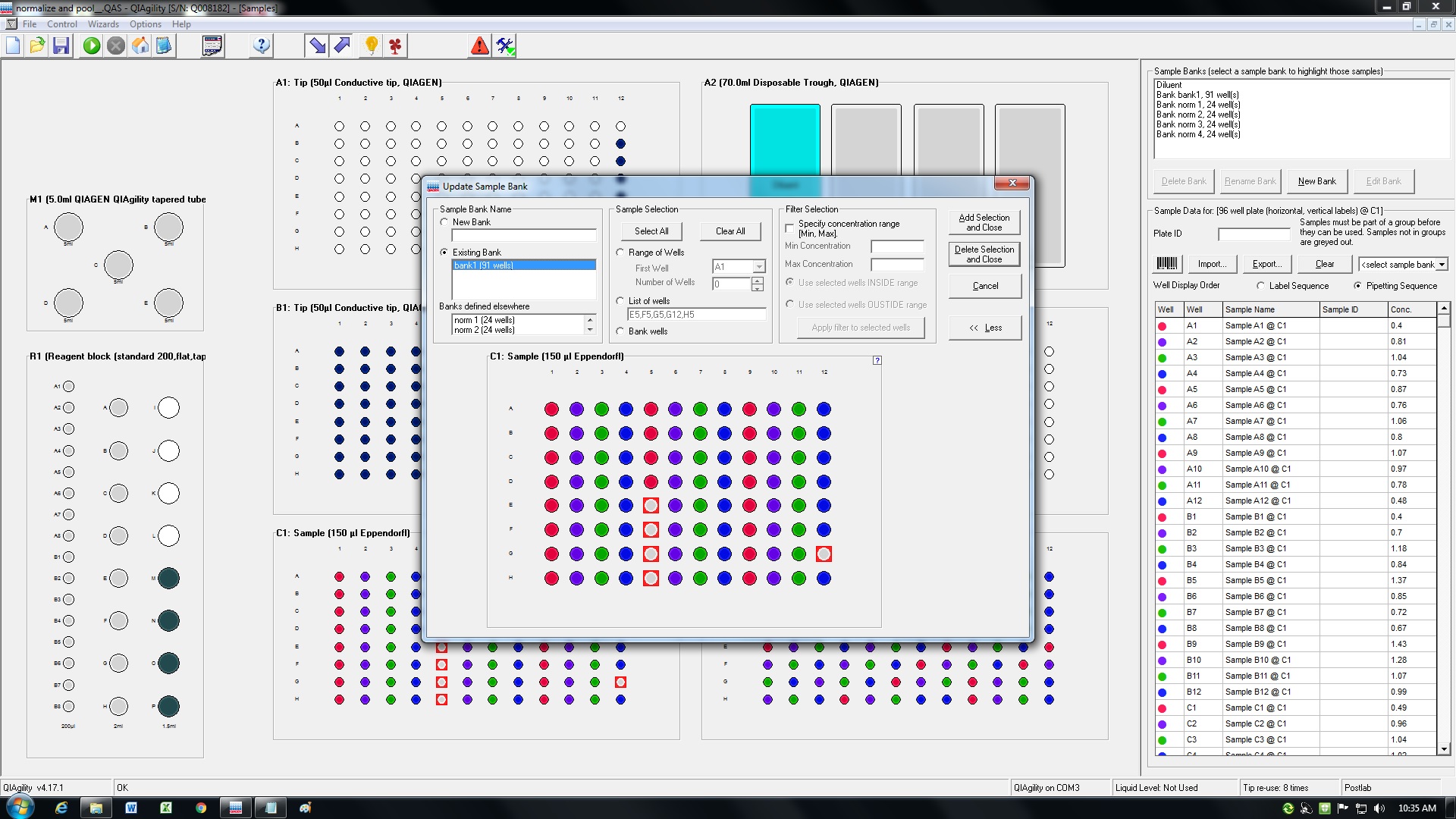
- Highlight area C1 by left clicking on it

- \Sample Data (right window)\Clear

- \Sample Data (right window)\Import...

- Select .txt import \Import\Finish

**4.6.2.1 Delete empty wells (optional)**



- Empty wells can be removed by right-clicking on area C1

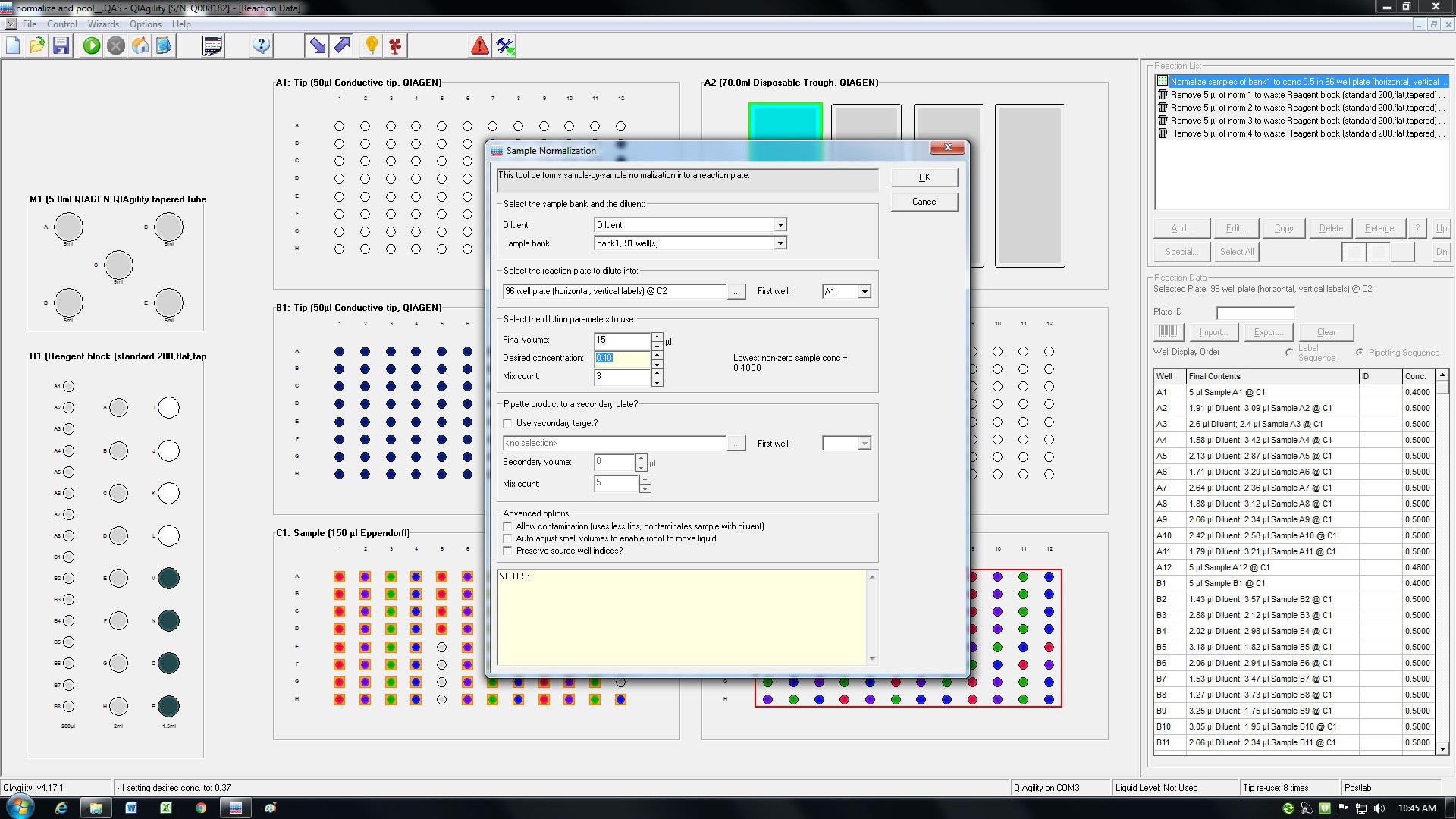
- \Remove selected wells from sample bank...

- In the window Update Sample Bank, select wells to be removed by left-clicking on them while holding down Ctrl

- In the field Existing Bank, select bank1 (.. wells)

- Click button Delete Selection and Close

**4.6.3 Sample normalization**

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- Highlight area C2 by left-clicking on it

- \Reaction List (top right)

- Double-click on Normalize samples of bank1 to conc...

- In the Sample Normalization window, adjust Final volume to the amount of available PCR product. If you have < 15μl left after the QIAxcel measurement(s), add 3μl to this number.

- Adjust Desired concentration to the Lowest non-zero sample conc (in this case 0.40)

- Press Tab to apply

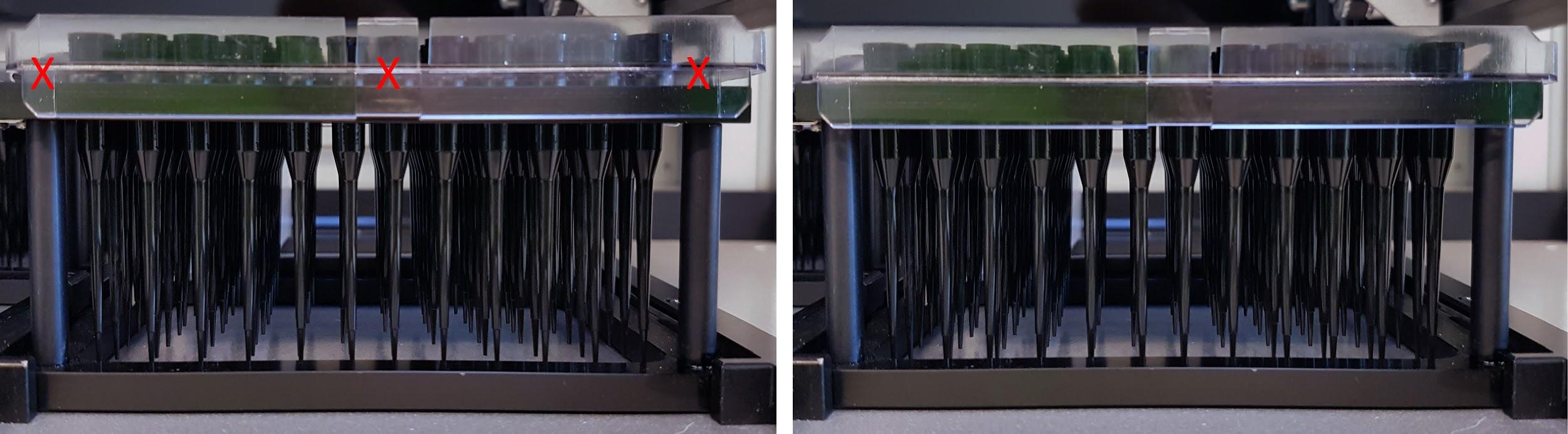
- The NOTES: field should be empty

**4.6.4 Refill tips and MQ**

- Wearing gloves, remove empty tip racks from the QIAgility and replace them with full racks.

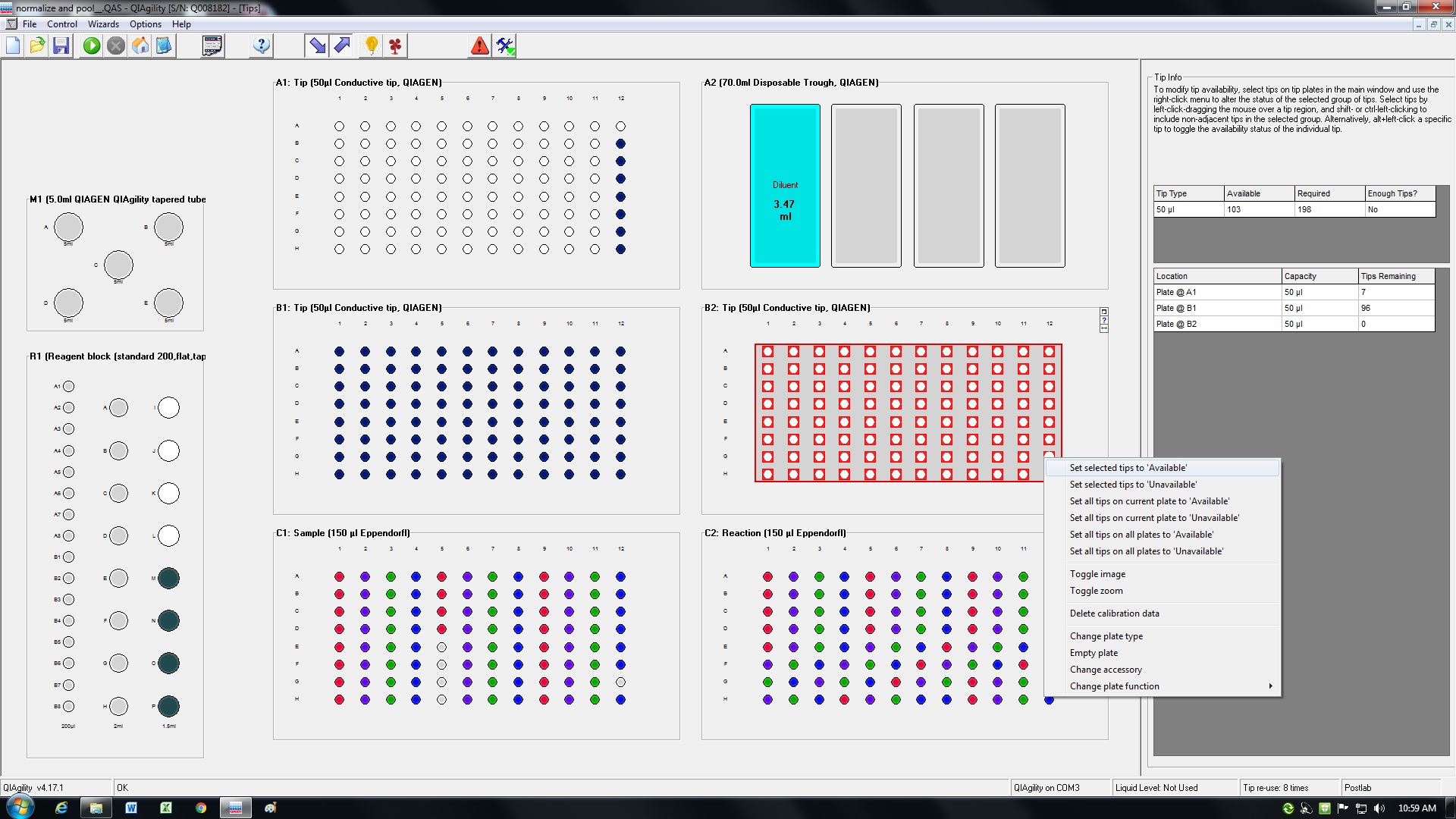


- When placing full racks in the QIAgility frame, make sure to align them correctly, so the notch in the plate aligns with the white dot on the frame



- Ensure the new tip rack closely connects with the frame by pressing along the ridges with 2 hands

- Replace the diluent and make sure the reservoir is half-full with MQ



- Select de wells in the refilled areas by dragging a field around them and right clicking

- \Set selected tips to ‘Available’

- Place 4 new Eppendorf low-bind 1.5ml eppies in the marked positions in area R1

- Place 2nd PCR plate in area C1 (Standard is Eppendorf twin.tec PCR plate 96 skirted. If you have a different plate type, highlight area C1 by left-clicking on it\right-click\Change plate type\Ok\)

- Place empty skirted PCR plate in area C2

- Click Start button 

- Check all boxes and click Run

**4.7 MN BEAD CLEANUP OF POOL**

- Let the MN beads get to room temperature (remove from fridge 15 min before use)

- Prepare fresh 80% EtOH (1ml per sample)

- Pool the contents of the 4 eppies together and measure the total volume by pipetting

- Mix the MN beads well by vortexing

- Add 0.9x (pool volume) MN beads to the pool and vortex

- Incubate at room temperature for 5 minutes

- Place the plate on the magnetic eppie rack

- When the solution is clear, carefully remove the supernatant (LEAVE THE PLATE ON THE MAGNET)

- Wash the beads 2x with 500µl 80% ethanol (LEAVE THE PLATE ON THE MAGNET)

- Let the beads air dry for 1 minute (LEAVE THE PLATE ON THE MAGNET)

- Take the plate off the magnet and resuspend the beads with 300µl MQ, by pipetting up and down

- Place the plate back on the magnet

- When the solution is clear, move 280µl of the the supernatant to a clean low-bind eppie

**4.8 MEASURE SUBPOOLS ON QIAXCEL**

**-**Measure the subpools in triplo on the QIAxcel

- Use the average molar concentration of the three measured subpools for pooling of these pools

**4.8.1 Pooling of pools**

If equimolar pooling of different pools is required, follow this calculation to dilute the pools to the lowest concentration.

example: pool Ⅰ = 100 nM

pool Ⅱ = 120 nM

pool Ⅲ = 250 nM

Adding an equal volume of the three pools together will give the right end concentration of 100 nM if pool Ⅱ and pool Ⅲ are also diluted to 100 nM.

Diluting pool Ⅱ to 100 nM = 120:100=1.2 So dilute pool Ⅱ 1.2 times (e.g. = 50µl pool Ⅱ + 10 µl MQ)

Diluting pool Ⅲ to 100 nM = 250:100=2.5 So dilute pool Ⅲ 2.5 times (e.g. = 50µl pool Ⅲ + 75 µl MQ)

Add an equal volume of pool Ⅰ, diluted pool Ⅱ and diluted pool Ⅲ together to create the 100 nM end pool.

**4.8.2 Pooling of pools with a different number of samples**

example: pool Ⅰ: 50 samples = 100 nM

pool Ⅱ: 10 samples = 120 nM

pool Ⅲ: 20 samples = 250 nM

First adjust the molarity of the pools to the lowest molarity (100nM in this example):

example: pool I: Already 100nM

pool II: 120nM / 100nM = 1.2x dilution eg. 10ul MQ + 50µl pool II

pool III: 250nM / 100nM = 2.5x dilution eg. 75ul MQ + 50µl pool III

Now the molarity of the 3 pools is the same (100nM). To make the final pool of these 3 pools, you have to correct for the number of samples in each pool. To do this, pipet the amount of µl corresponding to the number of samples in that pool into a new tube. For this example:

pool I: 50µl

pool II: 10µl

pool III: 20µl

**4.8.3 Measure endpool on BIOANALYZER**

Measure the end pool in triplo in the following dilutions: 3x 1:10 . 3x 1:100 and 3x undiluted

- Let the High Sensitivity Kit chemicals get to room temperature (remove from refrigerator 20 min before use)

- Fill cleaning chip with 350µl MQ and place in Bioanalyzer

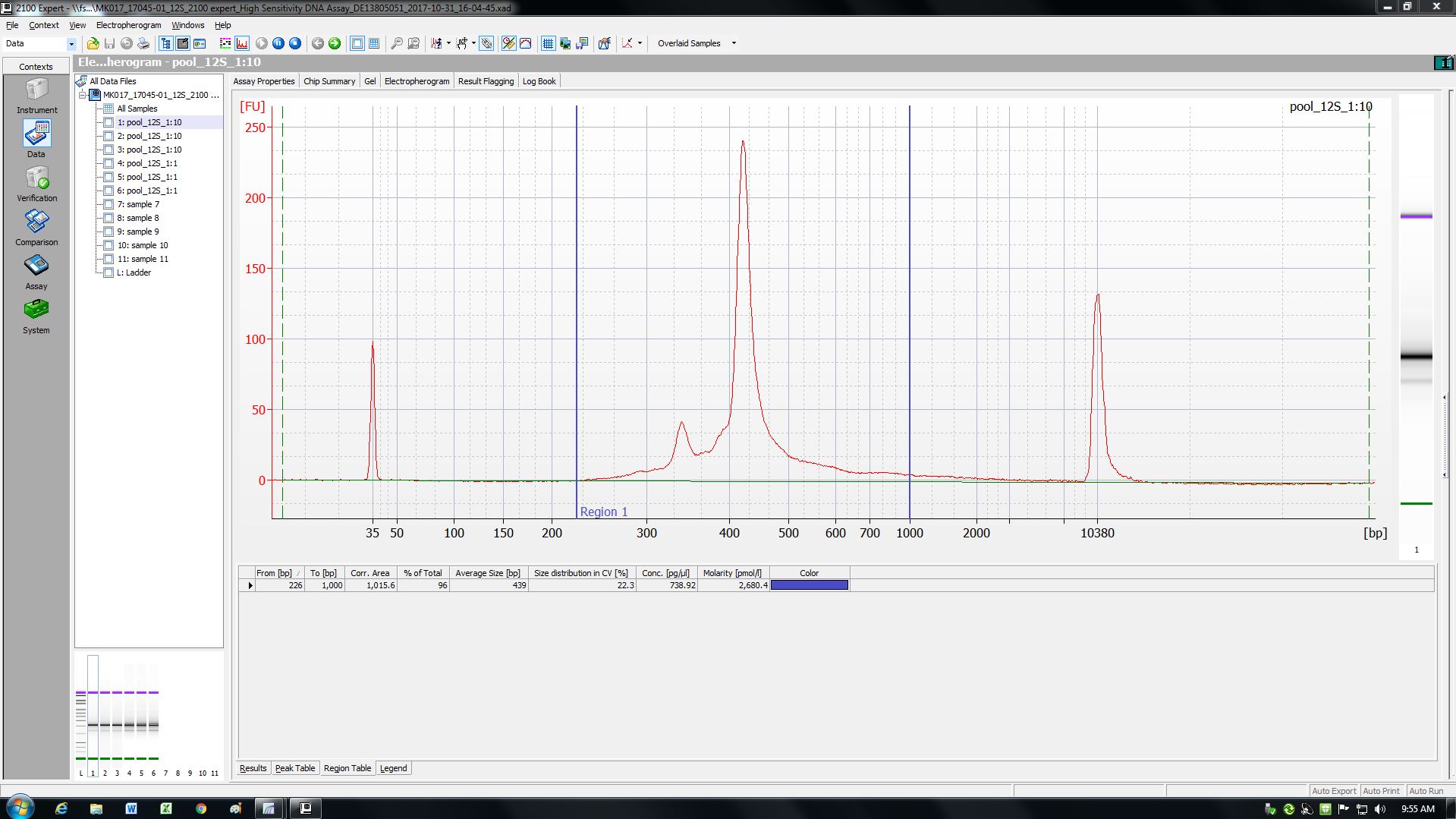
- Prepare a High Sensitivity Chip, according to the manual, which is available in the lab and can be downloaded here: [G2938-90321\_SensitivityDNA\_KG\_EN](https://drive.google.com/open?id=0B9VULIBkJsrEejYwY1dVOFJjVTA)

- Remove cleaning chip from Bioanalyzer

- Start Bioanalyzer software, indicate number of samples and provide sample names

- Place the High Sensitivity Chip in the Bioanalyzer

- Start run



- After run, select each sample by left-clicking on them (top-left)

- Tab \Region Table\ and adjust smear (blue lines) if necessary

- Write down the Molarity in pM [=pmol/l] for each sample of the same dilution and calculate the average values of the 1:10 and undiluted pool.

- Calculate the molarity of the pools in nM (in this example is the picture from the 10 times diluted sample meaning the concentration is 26.80 nM).

Safe the report as a pdf file.Baseclear needs 20ul of a minimum 5nM endpool

**5 ADDITIONAL PROCEDURES**

**5.1 For safety and lab regulations please reads the following documnets:**

[**PROC002\_GENERAL\_LAB\_REGULATIONS**](https://docs.google.com/document/d/1UeSiui7Q3syypLf-im2CqjScz03l6gZ12DW3qYKCMt0/edit)

[**PROC003\_SAFETY\_PROCEDURES**](https://docs.google.com/document/d/1dbvBB0RMZwp4_PpCMMdox2BsEsNK2zE2PrPyIYMy3bY/edit)

[**WI\_WS001\_PRE-LAB**](https://docs.google.com/document/d/11cxcmccdvFGDZH6deyfvllZMDWigP0stXIX5_W38lvk/edit)

[**WI\_WS\_NGS\_LAB**](https://docs.google.com/document/d/11soX2ez0UWM7cdl0a5jOx5nZrYK0Lm7zR6nMaLMA7_g/edit)

**6 CALCULATIONS**

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**7 QUALITY CONTROL**

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**8 RELATED/ASSOCIATED DOCUMENTS**

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**9 LITERATURE**

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