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1 As specified in Annex I

2 i.e. name of the person(s) responsible for the preparation of the document

3 Short name of partner(s) responsible for the deliverable

4 The Technical Annex of the project provides a list of deliverables to be submitted, with the following classification level:
Pub - Public document; No restrictions on access; may be given freely to any interested party or published openly on the web, provided the author and source are mentioned and the content is not altered.
Rest - Restricted circulation list (including Commission Project Officer). This circulation list will be designated in agreement with the source project. May not be given to persons or bodies not listed.
Int - Internal circulation within project (and Commission Project Officer). The deliverable cannot be disclosed to any third party outside the project.

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6 Two digits separated by a dot:
The first digit is 0 for draft, 1 for project approved document, 2 or more for further revisions (e.g. in case of non acceptance by the Commission) requiring explicit approval by the project itself;
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Abstract

High throughput DNA editing requires automation since it entails hundreds and often thousands of pipetting operations and track keeping of a comparable number of reagents. The work on deliverable 3.3 has focused on development and optimization of advanced scripts for automated execution of these complex operations which are required in DNA editing.
The scripts are written in 'Roboease' (A language developed by CADMAD) which enables us to command many robot liquid handling operations in very few lines of code. The purpose of these scripts is to make the process of writing and debugging Evoware programs (Tecan robot commands) shorter, easier and amenable to automation.
We have written a software module in Matlab that generates a series of Roboease scripts according to a DNA building plan.

Keywords[7]:

CADMAD, Evoware programs, advance scripts, DNA editing

1. Introduction

a. Aim / Objectives

The aim of this deliverable is to produce scripts for advances DNA editing operations to be executed by a Tecan Robot

b. State of the Art

Liquid handling robots are exclusively used as "one trick ponies" in the sense that they are calibrated to execute one task (one script) repeatedly. For example, they are often used for preparing samples for gel running or PCR. The challenge, then, is to run many different scripts on the same robot consecutively while keeping its precision, fidelity and robustness.

c. Innovation

We have written various scripts for DNA editing and calibrated them carefully to a point that we are now able to execute the entire spectrum of scripts required for DNA editing on the same robot using different configurations that are tailored to the requirements of each script.

2. Implementation

The process of developing a script begins with defining the requirements of the method that is to be automated (examining the protocol). After an analysis of the protocol we decide on the proper manner to automate it. This process includes determining the appropriate plastic-ware, tips, volumes, robot configurations etc. Then, a script is written and compiled in Roboease and its experimental testing begins. The script is then evaluated and an iterative process of improving and optimizing it begins, first with water and then with the real reagents. This includes changing the robot configurations (liquid

[7] Keywords that would serve as search label for information retrieval

classes), volumes and even the entire strategy of automating the script if a major problem is encountered.
Once the script is tried and tested we use it routinely and monitor its performance.
Partners ETHZ and Weizmann have implemented the following partial list of protocols for molecular biology (DNA construction) and biochemistry (assays for characterization) on a Tecan robot. This is a partial, exemplary list, in practice many more were developed and are in routine use:

- DNA purification
  - from PCR
  - plasmid purification from bacteria
  - plasmid purification from yeast
- DNA quantification
  - using Sybr Green in 384 wells
  - using 260/280 absorbance in special micro-channel plates
- Cell transformation
  - plasmid into *E. coli*
  - plasmid into S. cerevisiae
  - PCR product into *S. cerevisiae*
- Cloning
  - classical cloning (restriction digest, ligation, transformation)
  - isothermal assembly
  - yeast gap repair
- Diverse assays
  - beta-Gal assay (enzymatic detection of beta-galactosidase expression level) and similar assays
  - DNA – transcription factor interaction using fluorescence light polarisation (basic screening technology of BioVersys, a start-up company origination from ETHZ)
- DNA editing
  - Single molecule PCR
  - Gibson assembly
  - Phosphorilation of primers
  - Dilution of reagents
  - Preparing samples for plate Reader measurements
  - Preperation of samples for gel

To facilitate trouble-shooting of protocols, various quality control scripts were developed:

- Quality control of pipetting
- Pipetting of the same volume to each well (allowing for variations of pipetting parameters and source liquids)
- Dye pipetting and absorbance readout (each well holds varying dye volumes, but same total volume)
- Randomized pipetting (random choice of tip and volume for each well, and random sequence of wells)

The following analyses can be obtained from the data generated by the quality control scripts (see also D3.4 for a description of the low-level device drivers developed for these purposes):

Calibration of absorbance reader

Absorbance readers can be used to estimate liquid volume and (dye) concentration in a well. The readout value is proportional to the absorbance factor of liquid in the well and the distance the light travels through the liquid. If the liquid in the well had a flat surface, then the distance travelled through the liquid would be proportional to the volume in the well. However, since the liquid surface is curved rather than flat, the measurement depends on the position within the well at which the measurement is taken. Furthermore, surface curvature changes with liquid volume, liquid composition, well shape, and well coating, so the calibration procedure may need to be performed for many combinations of liquids, volumes, and plates.

Calibration of liquid level detection

Special pipetting tips can be used to detect the liquid level in a well. The tip is lowered into the well until a change in electrical resistance is detected. Due to the curvature of liquid surfaces, liquid level detection is subject to similar complications as the absorbance readouts described above. In addition, pipetting tips are lowered in discrete vertical steps (e.g., 0.1mm at a time), meaning that the point at which liquid is detected indicates a certain range of levels rather than an exact level.

Dispense volume consistency in multi-pipetting

There are two primary approaches to pipetting: a) liquid is aspirated from the source and then fully dispensed into the destination well, and b) a larger volume is aspirated from the source and then dispensed piece-wise into multiple destination wells. Approach b) will be referred to as multi-pipetting, and it is subject to additional degrees of inconsistency dependent on the initial aspirated volume and on the progression through the dispense sequence.

Detection of individual tip problems

Clogging, scratching, and bending of syringes and (fixed) tips will degrade their pipetting accuracy. Such problems can be detected by running pipetting routines that dispense specific volumes, which are then checked by the absorbance reader. If the performance of individual tips differs significantly from the overall average, then we have an indication that that tip should be further investigated.

Additional statistics grouped by various factors

Some pipetting inconsistencies can be very difficult to track down. These may be due to complex interactions between the properties of the liquid being dispense (e.g. viscosity, temperature), the pipetting parameters (e.g. speed of dispense, height of tip when dispensing), and plate type (e.g. well shape, well coating). For these cases, quality control scripts yield statistics that are grouped by such factors to allow the user to quantify the impact of changes they make while searching for a successful configuration.

3. Results

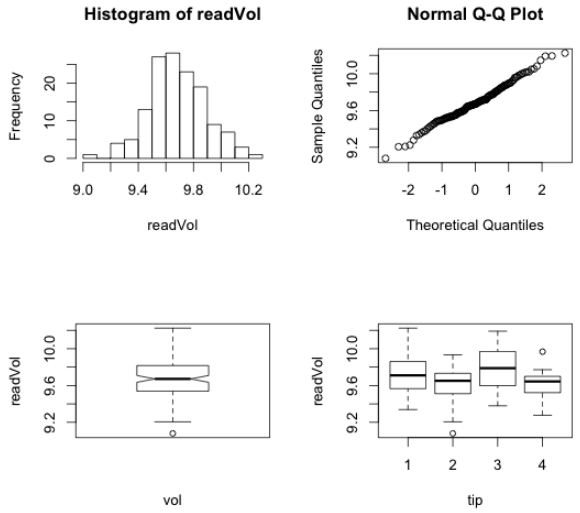
Biological protocols

The following protocols work reliably in the ETHZ laboratory so far: DNA purification from PCR, DNA quantification, cell transformation of plasmid into *E. coli* and yeast, as well as the “Diverse assays” listed above. Scripts for the other protocols exist, but the protocols themselves will require further optimization or troubleshooting before they can be used routinely.

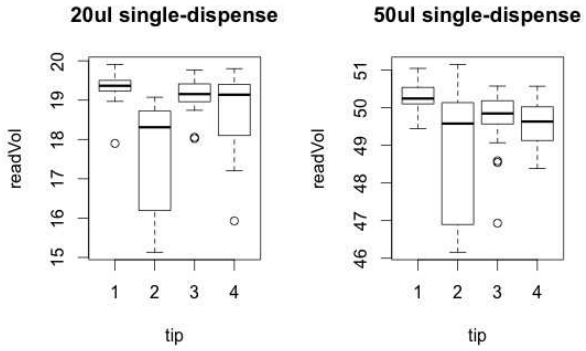
Quality control protocols

The quality control protocols have been successfully used for gathering statistics, ascertaining reliability, calibrating measurement equipment, detecting sources of error, and for altering the instructions sent to the robot in order to improve its performance.

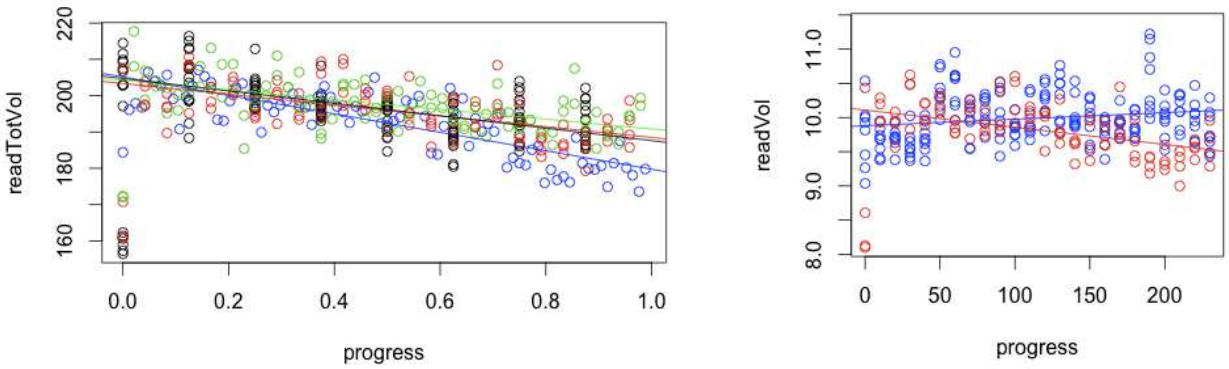
Ascertaining reliability: The graph below shows statistics for the case when the robot is instructed to dispense 10ul of liquid. The statistics reveal that the measured volume (labelled 'readVol' in the graph) is fairly normally distributed and biased towards volumes slightly smaller than 10ul. Analysis results such as this one can be used to automatically compensate for variability and biases in liquid handling (see below).



Detecting sources of error: The graph below shows boxplots of absorbance readouts for dye dispenses of 20ul and 50ul volume using four different, fixed tips. We see that tip 2 has much greater variability than the other tips. This allowed us to localize an important source of error and to correct it by replacing that tip.



Improving performance: The graph below on the left displays various measurements (runs, colour-coded) that show a consistent drop in dispense volume as multi-pipetting sequences progress. By adapting the pipetting instructions sent to the robot to take the recorded statistics into account, we are able to correct for some of the variability. This is shown in the graph on the right, where the red circles are from an uncorrected experiment and the blue circles are from the identical experiment with volume correction. The corrected experiment exhibits less than half of the variance of the uncorrected one.



The process of running automatic manufacturing scripts for the robot runs as follows:

Production of a Roboease script may be done in two manners: manually or automatically.
The automatic (and standard) way of creating a Roboease script is performed by running a Matlab module of the Tamuz system we developed. Roboease scripts are also often created manually. This is done whenever we have a special task that is not part of the automation system. For example: testing new hardware. Once a Roboease script is created we compile it using a Perl compiler we wrote into and Evoware executable file (a Tecan format). Executing the Evoware executable is done by loading it to the Tecan Evoware software and executing it with the robot.

A sample Roboease script for automating the Gibson assembly operation:

This script is newly developed and tested in Roboease. Its purpose is to automate the Gibson assembly reaction. It uses a function we wrote named AUT_GIB also written in Roboease.

This part of the script contains general information (like project name, date etc), marked by “DOC-ENDDOC”.
Not mandatory
############ DOCS ##############################
DOC
DNE Script
Project Name: GFPnoverWater25Run
Created on 06-Feb-2013 11:30:17
ENDDOC

This part of the script defines the robot deck configuration and is Mandatory. Tells the compiler which table template to use – each script may have a different robot deck configuration. Marked by “Table”.
############ TABLE ##############################
TABLE TABLE_DNE

This part of the script defines reagents and is very useful, but not mandatory
The structure of the command is (REAGENT) (REAGENT_NAME) (PLATE) (FIRST_WELL) (LIQUID_CLASS) (NUM_OF_WELLS)
#GLOBAL REAGENTS
REAGENT GIBSON_Mix_X2 T10 15 PIE_AUTBOT 1
REAGENT LB_SYBR T10 7 PIE_AUTBOT_SLOW 2
REAGENT DDW BUF12 1 PIE_TROUGH_AUTAIR 8

In this part we define various variables used in the script
DDW_VOL = 55
ELN_OLIGO_MIX_VOL = 4
Sample = 1.5
############ END OF DEFAULT HEADER ########################

Anything from here on is produced by the automation,
GIBSON_MIX_VOL = 5
GIBSON_SAMPLE_VOL = 5
################################

############ SCRIPT SECTION ################################
This part specifies the actual commands in the script to be executed.
In this case, we call a function named AUT_GIB with its parameters

SCRIPT
AUT_GIB P4:A1+3 P5:A1+3
Calling external procedure AUT_GIB
ENDSCRIPT

A procedure is a high level specification of several commands. In this case we built a procedure that specifies the operations required for the Gibson operation.
The structure of the external procedure AUT_GIB (for Gibson) is :

Function header
PROC Source_Loc Target_Loc

% Gibson step
# First command: distributing the Gibson mix to P4:A1+3. Mix after distribution.
DIST_REAGENT2 GIBSON_Mix_X2 Target_Loc GIBSON_MIX_VOL PIE_BIORAD_ACCUSURE TIPTYPE:AUTO,TIPMODE:KEEPTIP,MIX:PIE_MIX:5x7
Second command: transferring P4:A1+3 to P5:A1+3
TRANSFER_LOCATIONS Source_Loc Target_Loc GIBSON_SAMPLE_VOL PIE_BOTBOT_SLOW TIPTYPE:50

4. Conclusions

We have completed the writing and testing of numerous scripts and procedures like the example shown here for the Gibson operation. The next step is to use these scripts in the in production of the CADMAD libraries. In the future we are planning to focus on improving the maintenance of these scripts with the aid of our newly developed robot QA web system that is reported in WP4.