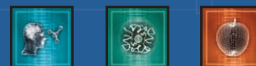
Recovering samples from Biacore® 3000 for mass spectrometry



Introduction

Biacore® 3000 Control software includes a microrecovery function designed for recovering material from the sensor surface in a very small volume (3-7 µl).

This note provides guidelines and recommendations for using this function to integrate Biacore analyses with mass spectrometry (MS).

Microrecovery

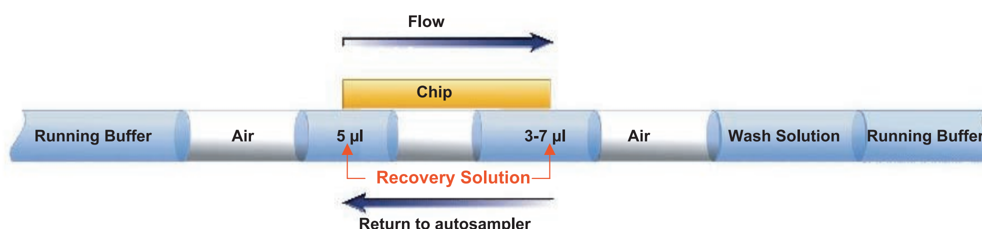


Figure 1
The principle of recovery of bound sample from the sensor surface.

Microrecovery in Biacore 3000 operates by injecting a series of small liquid volumes separated by air bubbles over the sensor surface.

Three liquid segments are used:

- Wash solution (20 µl) to rinse running buffer from the tubing and sensor surface.
- Recovery plug (3-7 µl of recovery solution) to elute the bound analyte from the surface.
- An additional segment of recovery solution (5 µl) to prevent contamination of the recovered sample with running buffer. This segment does not come into contact with the sensor surface.

The command washes the flow cells with a user-defined solution and injects a plug of regeneration solution (sandwiched between air bubbles) onto the sensor surface. The liquid flow is stopped for a user-specified length of time while the recovery solution is in contact with the sensor surface. The flow direction is then reversed and the recovery solution containing eluted analyte is dispensed into a vial. The volume of recovery solution is sufficient to cover all four flow cells in multi-channel mode.

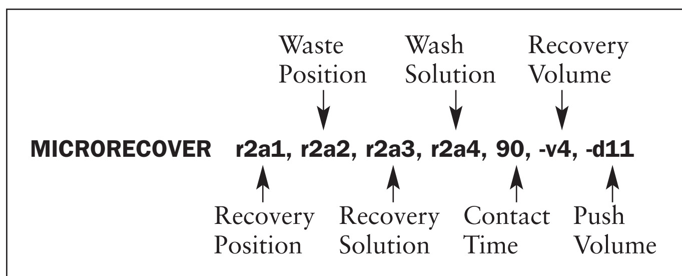
BIACORE

Biacore 3000 microrecovery command

The MICRORECOVER command has been specifically designed to allow recovery of ligand-bound analyte in a small volume suitable for further analysis.

This command can only be used from a pre-programmed text method in Biacore 3000.

Incorporation of the command into a method is shown in appendix A. This method is intended as an example and parameters should be altered to suit specific applications. The different vial positions, their function and command options are described below.



- **Recovery Position** is the position to which the recovered analyte will be delivered.
- **Waste Position** is a separate rack position specified for waste, instead of the normal waste position on the connector block, to eliminate contamination of the recovered sample with residues from the connector block.
- **Recovery Solution** is the regeneration solution used to remove the ligand-bound analyte.
- **Wash Solution** removes unbound contaminants from flow cells.
- **Contact Time** specifies the length of time the recovery solution remains over the flow cell(s). This time is user specified and interaction dependent. Ligand stability should also be considered when specifying this parameter.
- **Recovery Volume** is the volume of recovery solution, in which the ligand-bound analyte is eluted. The default recovery volume is 4 µl. Other volumes (range 3-7 µl) may be specified using the –v option with the command.
- **Push Volume** is the volume with which the dispenser pump is filled, in order to push the recovery solution over the flow cell(s) containing bound analyte. The default push-volume is 12 µl. Other volumes (10-21 µl may be specified using the -d option with the command.

Microrecovery sensorgram

During the Microrecovery event, the following actions take place:

The dispenser pump pushes the first air-bubble followed by the recovery plug into the flow cell. After a few seconds, the flow is stopped and the recovery solution stays in the flow cell for the user-specified contact time. Next, the flow is *reversed*, which means that the recovery solution is pulled out again, and the air-bubble that passed over the flow cell initially, is pulled back into the flow cell.

Because microrecovery includes air segments to keep the solutions separate and the flow is stopped during the recovery step, the sensorgram during the actual recovery does not contain much useful information. However, the amount of analyte removed from the surface can be read from the difference in response before and after the recovery step.

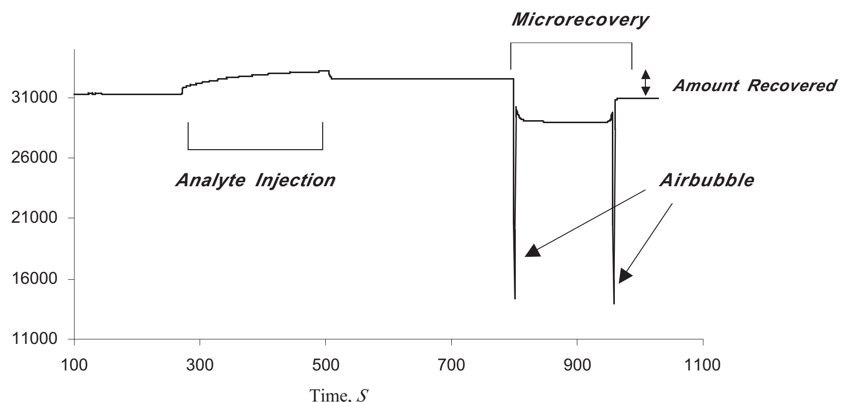


Figure 2
Example of a Microrecovery Sensorgram

Experimental design – a cooperative venture

The microrecovery experiment is designed to optimise mass spectroscopic sample processing and detection. Several of the parameters included in the following section require input from the mass spectrometrist.

Recovery efficiency & analyte amount

Initially, it is important to consider the concentration of the samples likely to be recovered and their suitability for MS analysis. It is useful to calibrate or check mass spectrometer sensitivity prior to Biacore sample preparation through MS analyses of a serial dilution series of standard solutions of the molecule of interest, or of a range of molecules of varying molecular weights and properties if the molecule of interest is an unknown. Bound analyte is recovered from the sensor surface in a volume of 3-7 µl using the microrecovery function.

How much analyte is there on the surface?
For one flow cell in Biacore 3000, a response of 1 RU corresponds to roughly 1 pg of bound analyte (for proteins and peptides). Thus the relative response may be converted to an approximate molar amount according to:

$$\textbf{amount bound (pmol)} = \frac{\textbf{relative response (RU)}}{\textbf{molecular weight (dalton)}}$$

Example: A relative response of 2,500 RU for an analyte with molecular weight 50,000 corresponds to approximately 2,500/50,000 = 0.05 pmol = 50 fmol.

In practice, recovery of 50% of the bound analyte is expected, although the efficiency is highly dependent on the analyte and experimental conditions. Recovery efficiency may be improved under favourable conditions; however, loss of analyte through adsorption to the fluidics, Teflon tubing and vials is inevitable.

MS analysis using MALDI or Q-TOF typically requires a minimum of 50-100 fmol of sample in the recovered volume. Under optimal conditions, analysis of amounts down to 10-20 fmol may be possible. The amount recovered may be increased via use of multiple flow cells and/or multiple recovery cycles. Combination of samples and concentration by chromatography techniques such as nanoscale reverse phase chromatography(e.g. ZipTip$_{\mu\text{-C18}}$ (Millipore) or lyophilization techniques may help to increase the overall concentration.

Recovered sample composition

Several factors will determine the recovered sample composition:

Recovery buffers are chosen for their compatibility with the experimental objective, the protein under examination and the chosen MS approach (eg MALDI vs electrospray). In general, volatile acids or bases should be used for recovery of analyte from the sensor chip.

If the molecule is to be examined in its intact form, it is essential that the chosen recovery buffers are compatible with the ionisation technique employed by the mass spectrometer. For example, MALDI and electrospray often place different restrictions on what buffers may be used. Buffer components such as salts, alkali cations, detergents, chaotropes, glycerol and solvents may hinder or interfere with sample ionisation and data analysis.

In certain cases, interaction analysis may require any or all of these different buffer additives for the particular protein system under examination; therefore, a balance or compromise should be made between efficient analyte recovery conditions and MS requirements.

In general, MALDI ionisation will be more tolerant of salts and other buffer additives than electrospray, however, it is advisable to minimise buffer components for both approaches.

Similarly, if the sample will be digested prior to MS analysis, a recovery buffer that facilitates

enzymatic digestion should be chosen. The properties of the particular enzyme chosen e.g. trypsin, pepsin, endo-Lys-C, or thermolysin should also be considered.

Often, protein denaturation is required prior to digestion, and a recovery buffer can be chosen that will assist with protein denaturation. In some cases, these reagents (chaotropes e.g. urea) may also enhance binding of molecule to reverse-phase column media.

Peptide finger printing by e.g. trypsin cleavage followed by ZipTip clean up improves the identification of the analyte. Sensitivity is generally 10 -100 times higher than for whole proteins with MALDI-MS.

Coordination of microrecovery experiments and MS analysis

The initial analyte recovery experiments should be performed *in situ* or immediately prior to MS analysis to avoid sample loss. The validity of this suggestion is protein dependent and not always necessary; however, it will be helpful in the early stages of assay development, particularly if troubleshooting is required. Information such as recovered sample composition and approximate sample concentrations should be made readily available to the mass spectroscopist during MS analysis.

Practical guidelines

Instrument preparation

Instrument cleanliness is imperative for successful microrecovery experiments.

A cleaning routine based on a mock recovery using beta octyl glucoside is recommended prior to starting experiments. It is also important to clean the connector block via removal and thorough rinsing with deionized water. The autosampler tubing is cleaned using the MIX command, setting a mix volume of 450 µL and placing a vial containing 50% ethanol in the appropriate rack position. Finally the syringe pump barrels and syringe tips should be inspected to ensure that they are clean and leak-free.

A microrecovery performance method is shown in Appendix B. This blank micro-recovery run is used to assess instrument performance for the microrecovery function.

Sensor surface preparation

Ligand density

The maximum analyte binding capacity (R_{MAX}) of the sensor surface is a function of the amount of ligand attached to the surface (R_L) and the relative sizes of ligand and analyte molecules (MW_A, MW_L):

$$R_{MAX} = MW_A / MW_L \times R_L \times S_m$$

where S_m, is the molar valency of the immobilised molecule.

Preparation of surfaces with **high ligand immobilization levels** increases the analyte binding capacity. High-density ligand surfaces can also increase analyte re-binding during complex dissociation, which may be helpful when fast complex decay is anticipated.

Flow cell configuration

It is important that at least one flow cell is a designated control surface.

A microrecovery control sample from this flow cell serves two purposes:

(1) Allows for the generation of a buffer background mass spectrum.

(2) Allows for the identification of any non-specific binding effects.

The amount of ligand-bound analyte recovered is directly related to the number of flow cells employed in serial mode; therefore, microrecovery from multiple flow cells increases the concentration of analyte in the eluted sample.

Pilot Biacore experiments

Pilot Biacore experiments are performed, analyte resources permitting, to ensure efficiency of the microrecovery procedure.

Suggested experiments include:

A. A preliminary Biacore binding/regeneration experiment

B. A dummy/blank microrecovery experiment

C. Microrecovery/analyte re-injection experiment

Buffers

Running buffer

Avoid running buffers containing Surfactant P20 (Tween®). Residues of Tween tend to contaminate micro-reverse phase chromatography (µRPC) columns and reduce the efficiency of ionisation in MS analyses. If detergent is required in the running buffer, use 0.005% octyl glycopyranoside (OGP) instead.

If possible, use volatile buffer substances such as ammonium carbonate, ammonium formate or ammonium acetate to minimize the risk of carry-over of non-volatile salts to the recovered sample. Phosphate buffers should be avoided as they often reduce ionisation efficiency.

Recovery solutions

The **recovery** solution and **wash** solution selections should be based on cooperative input from both the mass spectroscopist and the Biacore operator. Additionally, the recovery buffer concentrations should be as low as possible to minimize carry-over of recovery solution into the wash solution during microrecovery. Suggested recovery solutions and wash solutions are given below. Water is not recommended as a wash solution, especially when using acids or alkali for regeneration.

As previously discussed, detergents should be avoided; however, if adsorption to the autosampler tubing, fluidics or vials is suspected, 0.005% OGP may be included to minimize adsorption of recovered analyte.

Suggested Recovery Solutions

- 0.5% TFA
- 5% Formic acid
- 10 mM Glycine
- 2% Acetic acid
- 20% CH_3CN/0.1% TFA
- 10 mM Ammonium Hydroxide
- HBS-N

Suggested Wash Solutions

- 10 mM ammonium carbonate
- Low concentration buffer (1-20 mM)
- Running buffer (+0.005% OGP)

Preliminary Biacore binding/ regeneration experiment

This experiment is carried out to establish:

Binding

The analyte is injected over the control and active surfaces. The analyte concentration is based on the estimated affinity of the interaction and sample availability. If a non-specific binding interaction to the control surface is observed, the analyte buffer should be adjusted to prevent this event from occurring in future experiments. Suggested remedies are adjustment of the analyte buffer pH or addition of 1.0 mg/ml carboxymethyl dextran (Fluka #27560).

Use a slow **flow rate** (e.g. 5µl/min) for analyte injection to prolong the contact time and reduce sample consumption. However, the smaller the flow rate, the longer the time needed to achieve a certain binding level. Higher flow rates should be used if analyte supply is abundant. If possible, the analyte should be concentrated to improve binding, especially for low affinity analytes. This is particularly useful for rapidly dissociating analytes.

Regeneration

The intended recovery solution should be injected to ensure adequate regeneration of the surface. If regeneration of the binding interaction is incomplete, additional combinations of analyte and recovery solutions should be evaluated until a solution that successfully clears the surface of bound analyte is found. Testing the performance of recovery solution by using the INJECT command may not be sufficient since conditions during INJECT (constant flow) are more favorable than during MICRORECOVER (stopped flow). Always double check recovery efficiency by running MICRORECOVER.

It is helpful to include a cycle of analyte injection followed by injection of the intended wash solution to ensure that the wash solution **does not** significantly

regenerate the surface. (REMEMBER: the wash solution precedes the recovery plug in the microrecovery sequence).

At this stage, it is possible through examining the analyte binding curve and using BIAsimulation to determine the following parameters, for optimal microrecovery:

Analyte concentration in relation to binding affinity

For a 1:1 interaction at equilibrium, 50% of the ligand sites are occupied when the analyte concentration is equal to the dissociation constant, K_D. To achieve analyte binding approaching the maximum binding capacity, analyte concentrations should be 10-100 times the K_D value, or higher. For example typical K_D values for receptor-ligand interactions are in the range 1-10 nM, so analyte concentrations should be of the order of 100 nM or higher.

Analyte contact time

The time required for an interaction to reach equilibrium is determined by the analyte concentration and the dissociation rate constant. Slower dissociation rates demand longer contact times to reach a steady state (equilibrium) response. The table below illustrates times required to reach steady state for different combinations of analyte concentration and K_D values.

	k_d			
Concentration	10^{-1}	10^{-2}	10^{-3}	10^{-4}
K_D	34 sec	6 min	57 min	9.6 hrs
K_D10^1	–	63 sec	10.5 min	105 min
K_D10^2	–	–	68 sec	11 min

If analyte dissociation is fast, temperatures below room temperature may be used to slow analyte dissociation, thus increasing recovery efficiency.

Preliminary blank microrecovery experiment

A blank microrecovery experiment should be run to ensure that optimal microrecovery parameters have been chosen. This experiment does not require any analyte injections; however, the recovery solution, wash solution and ligand derivatized sensor surface chosen should be employed here.

This experiment verifies that the optimum push volume parameter (-d) in the micro-recovery command has been chosen.

Push-volume optimization

Optimal volumes for the –v and –d options may vary for given buffer conditions and between individual instruments. To establish whether the push-volume is appropriate for your instrument, the air segments preceding and following the recovery solution should be examined in the sensorgram. Air should be absent for the duration of the recovery contact time.

If air is present in flow cell 4 but not flow cell 1 during the recovery contact time, increase the value for the –d option.

If air is present in flow cell 1 but not flow cell 4 during the recovery contact time, decrease the value for the –d option.

If air is present in both flow cell 1 and flow cell 4, both of which indicate a fault in the experiment, or if it is not possible to fill all four flow cells by adjusting the –d option, increase the –v option.

If push-volume optimisation is required, the method given in Appendix C can be used to visually determine an appropriate push-volume.

Microrecovery & analyte re-injection experiment

Re-injection of the recovered sample immediately following microrecovery is used to verify analyte recovery and to quantify the amount of analyte recovered.

The recovered sample buffer is neutralised or diluted with an appropriate buffer to negate any effects of the recovery solution on the binding interaction.

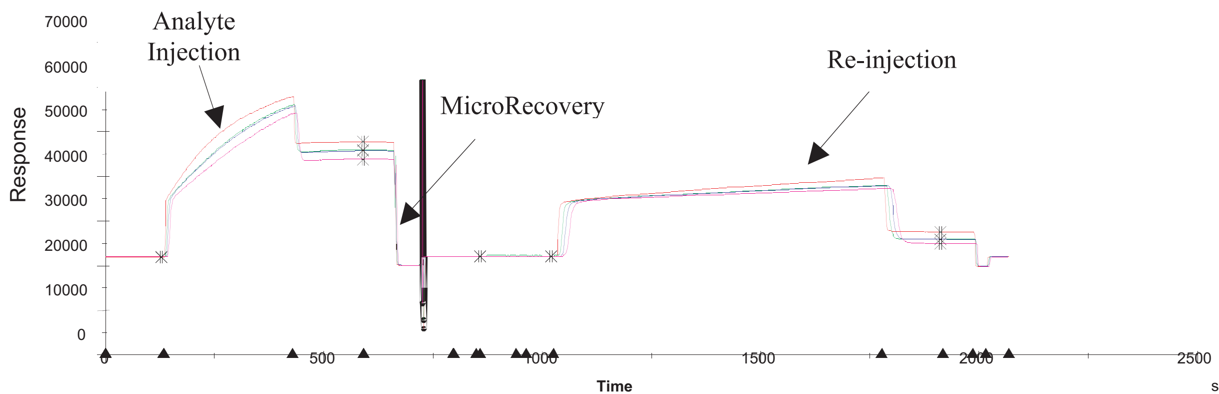


Figure 3
The re-injection binding response can be used to quantify approximate recovery efficiency.

The re-injection binding response can be used to approximate the recovery efficiency.

The analyte binding activity following exposure to the recovery solutions and loss of material due to re-adsorption to the fluidics, needle, tubing etc., should be considered in calculating recovery efficiency.

Note that the minimum concentration of analyte needed to detect binding in a Biacore instrument can vary with the kinetic parameters of the interaction. In some cases, analyte concentration in the recovered sample may be insufficient for detection by re-injection. The negative result of re-injection, therefore, does not necessarily mean that microrecovery has failed.

The microrecovery experiment

When suitable experimental parameters have been chosen for:

1) Recovery buffer, wash buffer and running buffer
2) Flow cell configuration
3) Analyte concentration
4) Analyte injection time
5) Recovery volume (-v; usually 3 or 4 µl)
6) Recovery solution contact time
7) Push-volume (-d; usually 10-12 µl)

a microrecovery method similar to that given in Appendix A can be assembled. This sample method takes into consideration some additional factors:

Further considerations:

Controls

Essential for any successful Biacore experiment.

The method given incorporates a blank flow cell recovery step. Mass spectroscopic analysis of this blank recovery sample allows any contamination of the recovered sample due to non-specific binding, protein adsorption or buffer carry-over to be assessed.

An internal control protein may also be added to the analyte solution prior to injection (e.g. 1µM insulin, human serum albumin or β-lactoglobulin). Evidence for these proteins in the mass spectrum may be used as an indication of non-specific binding or adsorption of the protein to the IFC, autosampler tubing or needle.

Additional Washing Steps

It is important that the time between the end of the injection and the recovery operation be minimised to prevent excessive loss of analyte through dissociation. However, since carryover or contamination of the samples may severely limit mass spectroscopic analysis, it is recommended that several additional washing steps be added to the method:

a) Prior to analyte injection, wash the needle with 70% formic acid, by using the following command:

   Transfer R1F7 W 100

b) Follow the analyte injection with a BYPASSWASH command:

   BYPASSWASH R2b1, R2b2, R2b3

The **BYPASSWASH** command washes the IFC, but not the flow cells, with up to three user-defined solutions.

This will, however, fill flow cell 1 with a small amount of by-pass wash 3 solution, running at a negative flowrate of -12 µl /minute over 10 seconds (2 µl).

The command is intended primarily to prepare the microfluidics for high performance recovery of bound material using the MICRORECOVER command.

The flow rate should be set to zero directly after analyte injection. This will promote rebinding of the analyte in flow cells

Sample handling

The recovered analyte should be dispensed to a suitable vial for further processing.

For most biomolecules, plastic vials are preferable to glass since adsorption of analyte to the vial surface is reduced.

Losses of material through adsorption and sample handling can be very significant with the small volumes and low concentrations involved. Therefore, minimize contact of the recovered sample with all surfaces prior to MS analysis (i.e. avoid unnecessary pipetting, transferring from vial to vial, etc). Contact time or the time between sample recovery and MS should also be minimised.

Summary

The integration of Biacore 3000 and mass spectrometry will be a successful endeavor when details of experimental design and optimisation are considered carefully by both the Biacore operator and the mass spectroscopist. The recommendations provided in this technical note are intended as a starting point for researchers interested in accessing the microrecovery capability of Biacore 3000. To view an example of these principles in practice, see Application Note 6 – Ligand Fishing with Biacore 3000: Selective Binding, Recovery and MALDI MS of a Specific Interaction Partner.

APPENDIX A

Sample Microrecovery Method

```
------------
MAIN
PRIME
PRIME
APROG Recover
APPEND STANDBY
END

DEFINE APROG Recover
FLOW 5
TRANSFER R1F7 W 100                      ! Needle-Wash with 70%Formic Acid
FLOWCELL 1,2
INJECT r2b1 15
BYPASSWASH r2f5 r2f6 r2f7
MICRORECOVER r2c1 r2c2 r2a3 r2a4 90      ! Control Microrecovery
FLOWCELL 3,4
INJECT r2b1 15
BYPASSWASH r2f5 r2f6 r2f7
MICRORECOVER r2a1 r2a2 r2a3 r2a4 90 -v4 -d11
END
```

APPENDIX B

Microrecovery Performance Check

Method

The following test method is similar to that given in the *Instrument Handbook* and employs diluted BIAtest solution and de-ionised water.

```
-----------
MAIN
APROG Recovery_Check
END

DEFINE APROG Recovery_Check
FLOW 10
BYPASSWASH r2f5 r2f6 r2f7
MICORECOVER r2a1 r2a2 r2a3 r2a4 30
END
------------
```

Rack position definitions:

R2F5, R2F6, R2F7 : Bypasswash solutions : Desorb 1, Desorb 2, water.

R2A1/R2A2 : Empty tubes, R2A1 is where recovered solution is dispensed and R2A2 is for recovery waste collection.

R2A3, recovery solution, in this case diluted BIAtest solution (20-fold dilution to ~ 1000 RU)
R2A4, wash solution, in this example water (~ -2000 RU)

APPENDIX C

Push Volume Optimisation Method

```
------------
MAIN
PRIME
PRIME
PRIME
  APROG      d10
  APROG      d11
  APROG      d12
  APROG      d13
  APROG      d14
  APROG      d15
  APPEND     standby
END

DEFINE APROG d10
  CAPTION Microrecovery at Pushvolume d10
  FLOW 10
  BYPASSWASH r2f3 r2f3 r2f3
  MICRORECOVER r2a1 r2a2 r2f4 r2f5 30 -v4 -d10
END

DEFINE APROG d11
  CAPTION Microrecovery at Pushvolume d11
  FLOW  10
  BYPASSWASH r2f3 r2f3 r2f3
  MICRORECOVER r2a1 r2a2 r2f4 r2f5 30 -v4 -d11
END

DEFINE APROG d12
  CAPTION Microrecovery at Pushvolume d12
       FLOW       10
       BYPASSWASH r2f3 r2f3 r2f3
       MICRORECOVER r2a1 r2a2 r2f4 r2f5 30 -v4 -d12
END

DEFINE APROG d13
  CAPTION Microrecovery at  Pushvolume d13
       FLOW     10
       BYPASSWASH r2f3 r2f3 r2f3
       MICRORECOVER r2a1 r2a2 r2f4 r2f5 30 -v4 -d13
END

DEFINE APROG d14
  CAPTION Microrecovery at Pushvolume d14
       FLOW       10
       BYPASSWASH r2f3 r2f3 r2f3
       MICRORECOVER r2a1 r2a2 r2f4 r2f5 30 -v4 -d14
END

DEFINE APROG d15
  CAPTION Microrecovery at Pushvolume d15
       FLOW       10
       BYPASSWASH r2f3 r2f3 r2f3
       MICRORECOVER r2a1 r2a2 r2f4 r2f5 30 -v4 -d10
END
DEFINE APROG d16
  CAPTION Microrecovery of HBS-EP Pushvolume d16
       FLOW       10
       BYPASSWASH r2f3 r2f3 r2f3
       MICRORECOVER r2a1 r2a2 r2f4 r2f5 30 -v4 -d16
END
```

References

1) Carsten P. Sonksen, Östen Jansson, Magnus Malmqvist and Peter Roepstorff, *Poster Presentation.“An Improved Method for Combining Biacore Instrument with MALDI Mass Spectrometry”*

2) Östen Jansson, Carsten P. Sonksen and Peter Roepstorff, *Poster Presentation,“BIA-MS of Small Molecules”*

3) Carsten P. Sonksen, Eckhard Nordhoff, Östen Jansson, Magnus Malmqvist and Peter Roepstorff; *Anal. Chem.* **1998**, 70, 2731-2736 *“Combining MALDI Mass Spectrometry and Biomolecular Interaction Analysis Using a Biomolecular Interaction Analysis Instrument”*

4) Biacore Application Note 6: Ligand Fishing with Biacore 3000 Selective binding, recovery and identification by MALDI MS of a specific interaction partner

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