Comparability Testing of Recombinant Cascade Reagents against Traditional LAL Reagents

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USP Open Forum, Nov 2021

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Disclaimer

- The information in this presentation is given for the purposes of education and discussion

- It is not intended to be, and it should not be used as, a substitute for the regulations or regulatory guidance

- Decisions and actions should be based on the relevant regulations, guidance documents and pharmacopeial chapters, not on this presentation

Outline

- Introduction to rCR
  - Where did rCR come from?
- Evaluation program
- Evolution of Comparability studies
  - Overview of what we have done so far
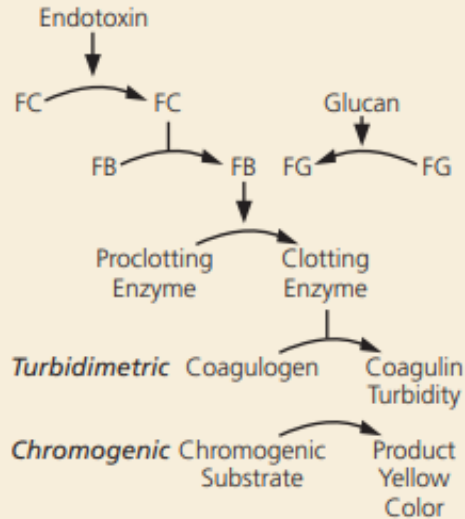 - New data
- What is next for rCR

Introduction to rCR

Recombinant Cascade Reagents (rCR)
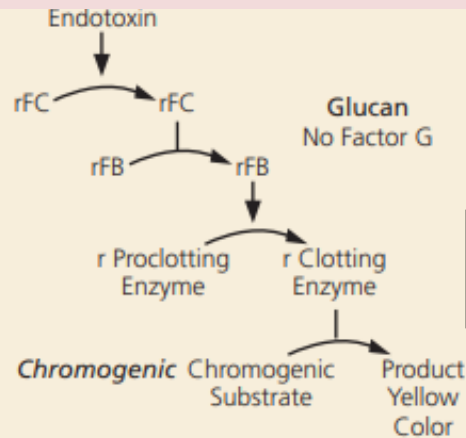
rCR is a recombinant reagent for kinetic chromogenic assays
Based on the genetic sequence of *Limulus polyphemus*

Per JP: two types of recombinant reagents

rFC   rCR

Uses the same detection method as LAL – absorbance

Recombinant Cascade Reagents (rCR)

The road to rCR

Seikagaku develops rCR clones

rCR included in collaborative studies[2,3]

Technical transfer

| 2010 | 2015 | 2017, 2018 | 2019 | 2020 | 2021 |

rCR launch in Japan[1]

rCR published on in a large suitability study[4] Evaluation studies

Global launch of rCR Next generation

[1] **Mizumura et al. 2017**
[2] *Kikuchi et. al., 2017:*
[3] *Kikuchi et. al., 2018:*
[4] *Muroi et. al., 2019:*

Comparability studies

rCR is based on 10 years worth of research, 6 years worth of data
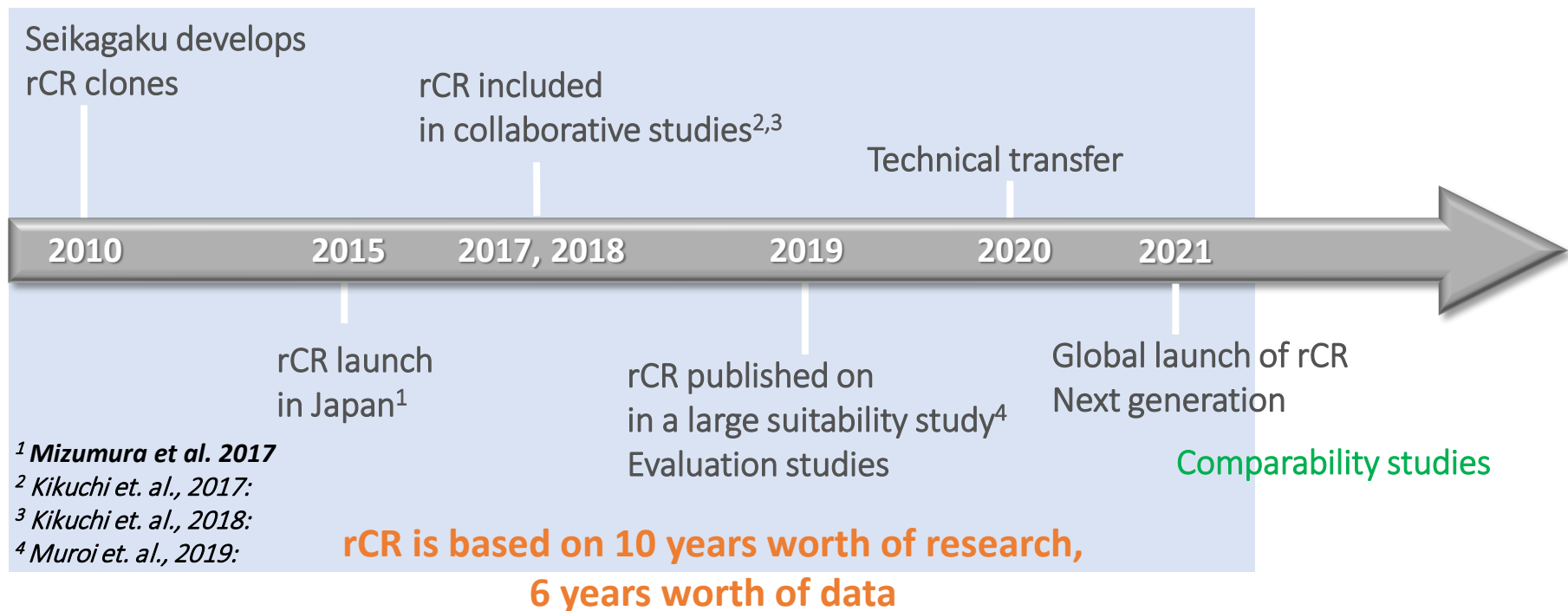
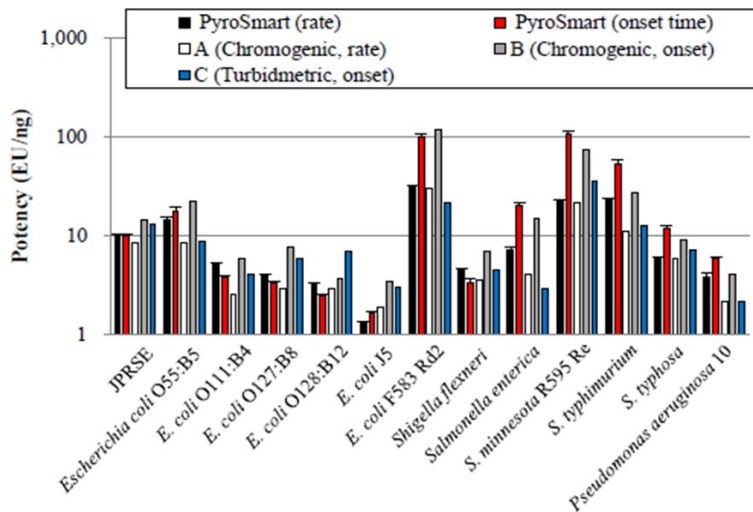# Recombinant Cascade Reagents (rCR)

The road to rCR

- Published in four peer-reviewed publications:
  - Mizumura et al. 2017
  - Kikuchi et al. 2017
  - Kikuchi at al. 2018
  - Muroi et al. 2019
- Next publication which is specifically focused on rCR next generation is in preparation

Recombinant Cascade Reagents (rCR)

The road to rCR

Comparability of rRC

Muroi et. al., 2019:

Comparability of rRC next generation

CSEs	rCR	Mean LAL	Relative Recovery %
	Final potency	**Final potency**	
	EU/ng	**EU/ng**	**rCR / LAL *100%**
Pseudomonas aeruginosa 10	4.18	6.56	64
Salmonella thyphimurium	7.05	4.92	143
Salmonella minnesota R595	107.75	60.46	178
Serratia marcescens	4.13	4.02	102
Escherichia coli O55:B5	11.96	7.27	164

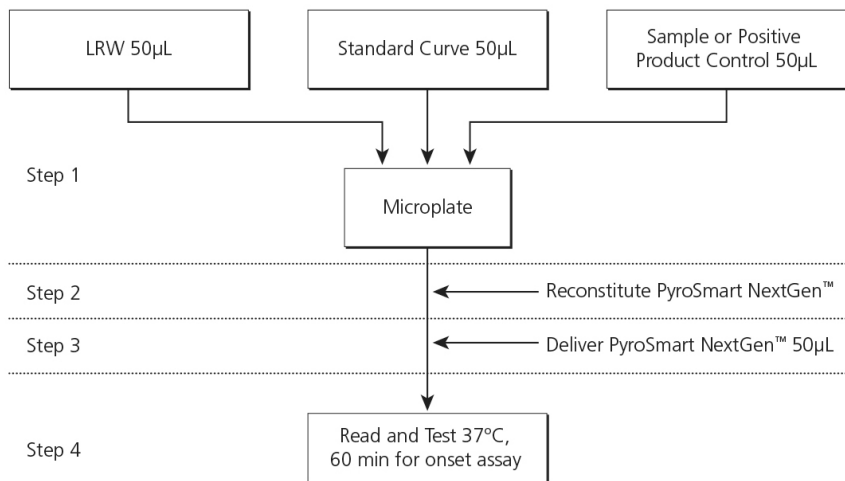*ACC Technical Note, 2021*

where $Relative\ recovery = \frac{rCR\ result}{LAL\ result} * 100\%$

The Workflow of rCR

Kinetic chromogenic assay

Absorbance plate reader

| LRW 50µL | Standard Curve 50µL | Sample or Positive Product Control 50µL |

Step 1 — Microplate

Step 2 — Reconstitute PyroSmart NextGen™

Step 3 — Deliver PyroSmart NextGen™ 50µL

Step 4 — Read and Test 37°C, 60 min for onset assay

Absorbance Tube reader

| LRW 200 µL | Standard Curve 200 µL | Sample and Positive Product Control 200 µL |

Step 1 — Reaction Tubes

Step 2 — Reconstitute PyroSmart NextGen™

Step 3 — Deliver PyroSmart NextGen™ 50µL

Step 4 — Incubate at 37°C and read during 80 minutes onset assay

Typical Results from rCR

STANDARD RESULTS

NC

IDENTIFIER	WELL	CONCENTRATION	ONSET TIME	AVERAGE ONS
NC1	1	N/A	>=3443 s	>=3423 s
NC1	2	N/A	>=3403 s	
SC1-1	3	0.001 EU/mL	2979 s	2993 s
SC1-1	4	0.001 EU/mL	3006 s	
SC1-2	5	0.01 EU/mL	1438 s	1421 s
SC1-2	6	0.01 EU/mL	1405 s	

RVE 1

Correlation Coefficient:	-0.998	Slope:	-0.28	Y-Intercept:	2.62

Onset Time — tion (EU_per_mL)

PRODUCT RESULTS

Tap Water
LOT NO. cwfi9
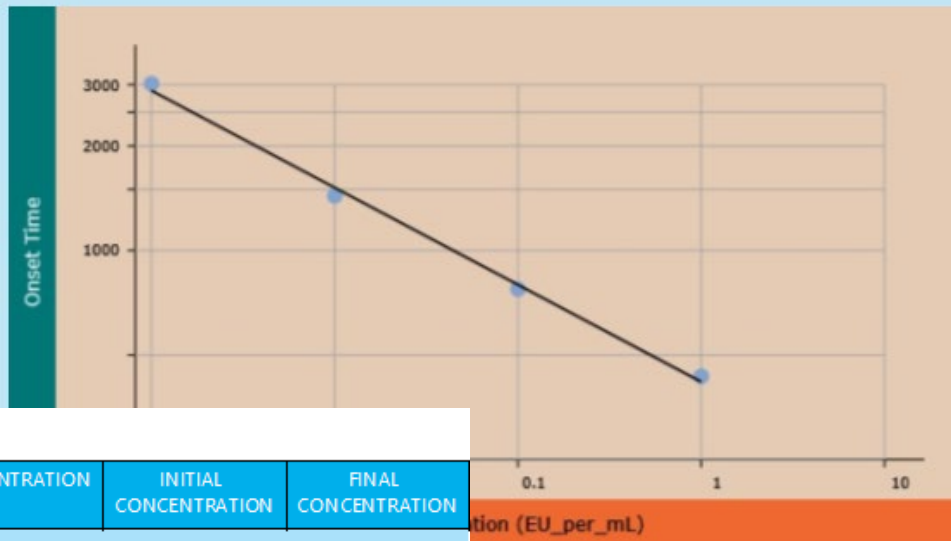
| IDENTIFIER	WELL	DILUTION	ONSET TIME	CV	AVERAGE ONSET TIME	CONCENTRATION	INITIAL CONCENTRATION	FINAL CONCENTRATION
P1-1	19	10	>=3193 s	⚠	>=3188 s	<0.001 EU/mL	1 mL/mL	<0.01 EU/mL
P1-1	20	10	>=3182 s					
COMMENT:								
P1-1+	21	10	801 s	0.00 %	801 s	0.0944 EU/mL	1 mL/mL	N/A
P1-1+	22	10	801 s					
PPC VALUE 0.1 EU/mL			PPC RECOVERY 94 %			PPC RECOVERED 0.0944 EU/mL CONCENTRATION		

Advantages of rCR over LAL

- Standardization
  - High reproducibility of results

- Specificity to endotoxin
  - Eliminates Factor G pathway

- Sensitivity and Speed
  - 0.001 EU/mL in 50minutes

- Sustainability
  - No dependence on horseshoe crabs
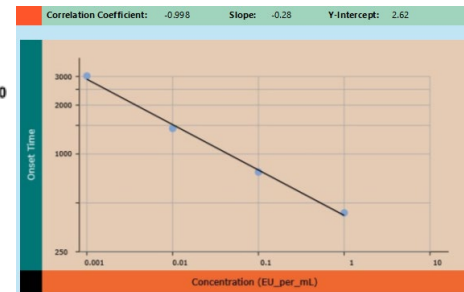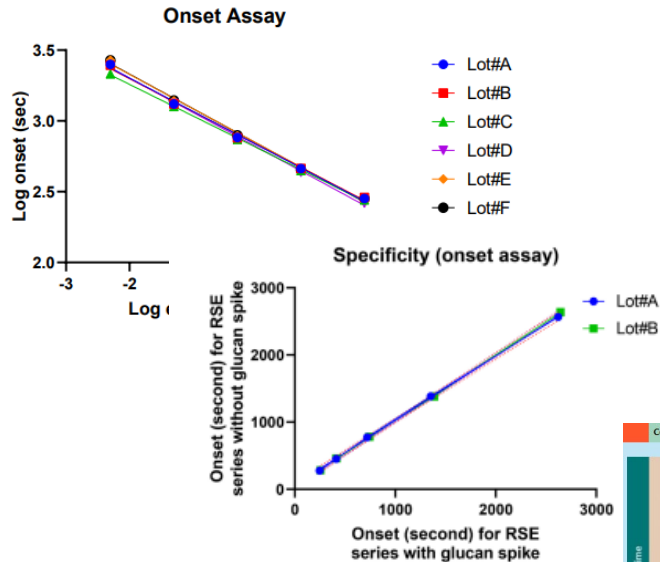
Advantages of rCR over LAL

Suitability for a wide range of products

- Finished drug products

Products	MVD λ=0.005 EU/mL	Non-Interfering Dilution (NID)		
		rCR	LAL - Ch	LAL - T
Sodium citrate injection for transfusion	1,120	1	4	4
Vancomycin HCl injection	5,000	64	32	128
Glucose injection	100	8	8	4
Heparin Ca	15,000	512	128	8
D-mannitol injection	100	2	4	2
Acyclovir 75mg/mL	N/A	4	8	8
Insulin 8mg/mL	N/A	1	2	1
PBS buffer	N/A	1	1	1
WFI	50	1	1	1

ACC Technical Note, 2021

Evaluation Program

rLAL Evaluation

- PyroSmart NG kit evaluation
  - Update from similar 2019 study presented at USP using original Seikagaku PyroSmart
- Experiment
  - 10 different pharmaceutical products; similar to Kikuchi, et. al. (2017)
  - 3 kinetic chromogenic LAL reagents
  - 4 recombinant reagents:
    - 2 end-point fluorescence rFC
    - 2 kinetic chromogenic rLAL
  - Used a common 0.01 EU/mL assay sensitivity, and a dilution and diluent optimized for rFC and 1 LAL
   - i.e. not necessarily optimized for all reagents

Unspiked Samples

Product	Units	LAL by Supplier			rFC/rLAL by Supplier			
		ACC	CRL	Lonza	ACC	BMX	Lonza	Seikagaku
Insulin Solution DP1	EU/100U	<20.0	<20.0	<20.0	<20.0	<20.0	<20.0	<20.0
Insulin Suspension DP1	EU/100U	<40.0	<40.0	<40.0	<40.0	<40.0	<40.0	<40.0
Monoclonal DP1	EU/mg	<0.0140	<0.00833	<0.00833	<0.00833	<0.00833	<0.00833	<0.00833
Monoclonal DP2	EU/mg	<0.167	<0.167	<0.167	<0.167	<0.167	<0.167	<0.167
Histidine	EU/mg	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100
Sodium Acetate	EU/mg	<0.00250	<0.00250	<0.00250	<0.00250	<0.00250	<0.00250	<0.00250
Glass Cartridge	EU/unit	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Rubber Stopper	EU/unit	<0.500	<0.500	<0.500	<0.500	<0.500	<0.500	<0.500
LAL Reagent Water	EU/mL	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100
Yeastolate 25%	EU/mg	**>4**	**0.0639**	**0.0687**	**<0.0400**	**<0.0400**	**<0.0400**	**<0.0400**

ACC – Associates of Cape Cod; CRL – Charles River Laboratories; BMX - bioMerieux

- All tested LAL formulations were susceptible to beta glucan false positive from Yeastolate
- All tested recombinants were more specific for endotoxins

Spiked Samples
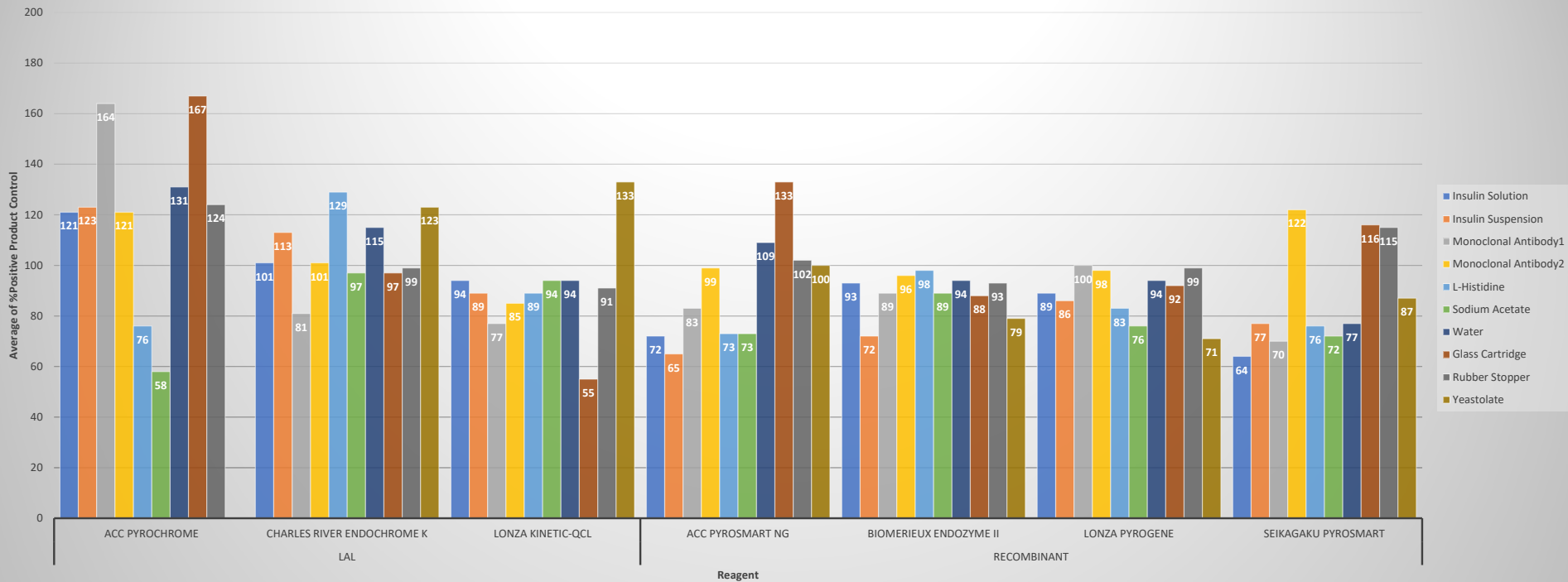
Spiked Samples

Sample	Recombinant		LAL	
	Average %PPC	%CV	Average %PPC	%CV
Insulin Solution DP1	80	17	105	13
Insulin Suspension DP1	75	12	108	16
Monoclonal DP1	86	15	104	41
Monoclonal DP2	104	12	96	10
Histidine	83	14	98	28
Sodium Acetate	78	10	83	26
Glass Cartridge	107	20	106	53
Rubber Stopper	102	9	105	16
LAL Reagent Water	94	14	113	16
Yeastolate 25%	84	15	128	6
Overall	**89**	**18**	**104**	**25**

- Recombinants and LAL are comparable
- Recombinants slightly more accurate: 90.9% PPC target due to hot spike (0.01 or 0.005 mL into 0.1 mL or 0.05 mL sample)
- Recombinants slightly more precise, however not all reagents are optimized

End User's Evaluations

Non-GMP products – tube reader

Sample	Dilutions	rCR Final conc. EU/mg	%CV	%PPC recovery	%CV	LAL - Ch Final conc. EU/mg	%CV	%PPC recovery	%CV	Relative Recovery % Mean rCR / Mean LAL *100%
	1:1000	3.236	0.43	97	2.58	5.324	1	104	1.47	
	1:2000	2.798	0.52	98	1.50	4.79	0.51	119	0.79	
Enzyme 1	1:4000	2.633	1.89	105	0.50	4.765	2.78	117	0.29	58%
	1:1000	1.392	3.25	96	3.7	2.493	0.51	113	2.68	
Enzyme 2	1:2000	1.183	1.40	98	0.06	2.501	1.43	113	1.64	52%

End User's Evaluations

Renal water – plate reader

Sample	Dilutions	rCR		LAL - T		Relative Recovery %
		Final conc. EU/mL	%PPC recovery	Final conc. EU/mL	%PPC recovery	
	1:5	3.57	137	2.31	162	
	1:10	3.8	98	2.88	85	
Lot 1	1:20	3.55	76	2.54	100	141%
	1:5	7.10	- 22	3.64	181	
	1:10	9.15	62	6.16	98	
Lot 2	1:20	7.84	131	8.39	125	140%
Lot 3	1:10	3.84	57	1.60	121	240%
Lot 4	1:10	11.4	26	4.23	160	Not calculated
Lot 5	1:10	< 0.1	87	< 0.1	98	N/A
Lot 6	1:10	0.324	97	0.138	106	234%
Lot 7	1:10	< 0.1	106	< 0.1	132	N/A

Screening for endotoxin within a manufacturing process

Sample	Dilutions	rCR				
		Final conc. EU/mL	%CV	%PPC recovery	%CV
Raw material – before purification	1:1,000	> 1000	N/A	N/A	N/A
Raw material – after the 1st purification step	1:100	43.2	4.86	87	1.41
After 2nd purification step	1:100	11.2	3.21	50	2.37

Evolution of ACC Comparability Study

Evolution of ACC Comparability Studies

Acceptance criteria

	Parameter	Criteria
Validity of test	Standard curve - linearity	R ≥ 0.980
	Intra assay precision – based on concentration	CV% ≤ 30%
	Negative control	Mean Onset Time of negative controls > 1.1 x Mean Onset Time of the lowest standard
Validity of sample	PPC recovery %	50 – 200 %
Comparability assessment	Relative recovery % = rCR / LAL x 100%	50 – 200 %

Evolution of ACC Comparability Studies

Non-sterile OTC drug products

	Non-Sterile Drug Products			Final conc. EU/mL at NID		
Sample No.	Application	API	Stock Solution	rCR	LAL Chrom	Relative Recovery %
1	Electrolyte	water, dextrose, ciric acid	N/A	< 0.05	< 0.05	n/a
2	Eye itch relief	ketotifen fumarate	0.035%	<5	<5	n/a
3	Antiseptic	benzalkonium Cl Lidocaine HCl	0.13% 2.5%	< 5	< 5	n/a
4	Skin cleanser	chlorhexidine gluconate	4%	NR		n/a
5	Allergy relief	difenhidramina HCl	18.5%	< 0.05	< 0.05	n/a
6	Ophthalmic sol	ciproflaxin HCl	0.3%	< 5	< 5	n/a
7	Eye drops	pheniramine maleate naphazoline HCl	0.315% 0.02675%	< 0.05	< 0.05	n/a
8	First aid antiseptic	Iodine Na iodine Alcohol	2% 2.4% 47%	< 5	< 5	n/a

Evolution of ACC Comparability Studies

Non-sterile OTC drug products

	Non-Sterile Drug Products			Final conc. EU/mL at NID		
No.	Application	API	Stock Solution	rCR	LAL chromo	Relative Recovery %
9	Itch relief	diphenhydramine HCl	2%	< 0.5	< 0.5	n/a
10	Saline laxative	saline laxative	N/A	< 0.5	< 0.5	n/a
11	Saline laxative	saline laxative	N/A	< 0.5	< 0.5	n/a
12	First aid antiseptic	benzalkonium cl	0.13%	< 0.5	< 5	n/a
13	Dry nose spray	Hyaluronate Na, Aloe vera, allantoin, CMC	N/A	0.49	0.9	54 %
14	Nasal mist	cardiospermum, glaphimia galuca, lufta operculate	N/A	< 0.05	< 0.05	n/a
15	Dry eye relief	polyethylene glycol 400 propylene glycol	0.4% 0.3%	< 0.05	NR	n/a
16	Lubricant eye drops	sodium carboxymethyl cellulose	0.25%	< 0.05	4.04	0 %
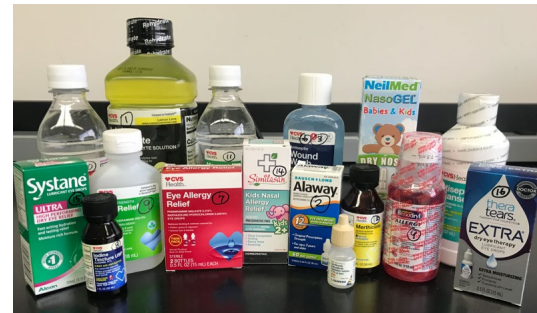
	Sample 16
	Final Glucan Conc. pg/mL
1:1,000	68,030
1:10,000	67,200

Samples containing a significant glucan background

- Case study
  - Product: cosmetic filler, primary component = carboxymethylcellulose (CMC)
  - BET method development
    - Tube reader
    - Turbidimetric LAL
    - Std. curve 0.1 to 0.001 EU/mL (with spiking at 0.01 EU/mL)
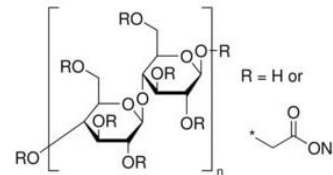
Unit	Dilution	Final conc. EU/mL	%CV	%PPC recovery	%CV	Reportable dilution (NID)
1	1:200	> 0.1	-	> 0.1	-	Invalid
	1:400	18.64	35.0	279	14.0	
	1:800	14.48	2.2	321	5.5	

Speaking of Glucans

Samples containing a significant glucan background

- Case study
  - Product: cosmetic filler (CMC)
  - BET method development
    - Tube reader
    - Turbidimetric LAL – ES
    - Std. curve 1 to 0.001 EU/mL
    - Spiking at 0.1 EU/mL

Unit	Dilution	Final conc. EU/mL	%CV	%PPC recovery	%CV	Reportable dilution (NID)
1	1:100	< 0.1	-	145	23	
	1:200	1.46	29	148	35	
	1:500	<0.5	-	192	20	
2	1:100	0.239	78	123	7	
	1:200	<0.2	-	149	6	Invalid
	1:500	<0.5	-	154	1.3	
3	1:100	0.148	40	151	12	
	1:200	<0.2	-	163	17	
	1:500	<0.5	-	148	3	

Speaking of Glucans

- ## Case study
  - ## Product: cosmetic filler
  - ## BET method development
    - ### Tube reader
    - ### **rCR**
    - ### Std. curve 5 to 0.005 EU/mL
    - ### Spiking at 0.05 EU/mL

Unit	Dilution	Final conc. EU/mL	%CV	%PPC recovery	%CV	Reportable dilution (NID)
1	1:100	< 0.5	-	96%	0.70	1:100
	1:200	< 1	-	97%	2.70	
2	1:100	<0.5	-	108%	0.04	1:100
	1:200	< 1	-	108%	3.10	
3	1:100	<0.5	-	94%	3.00	1:100
	1:200	<1	-	101%	1.60	
4	1:100	<0.5	-	80%	2.90	1:100
	1:200	<1	-	93%	0.83	
5	1:100	<0.5	-	90%	1.60	1:100
	1:200	<1	-	96%	1.00	

Evolution of ACC Comparability Studies

Comparability studies

- Deionized (DI) water samples
- *E. coli* culture

Sample	rCR Mean Final conc. EU/mL	LAL Mean Final conc. EU/mL	Relative Recovery % Mean rCR / Mean LAL *100%
DI water #1	8.965	6.563	137
DI water #2	1.354	1.498	90
DI water #3	5.260	5.607	94
DI water #4	1.190	1.055	113
DI water #5	0.063	0.053	119
DI water #6	0.238	0.202	118
DI water #7	0.037	0.043	86
DI water #8	1.718	1.485	116
DI water #9	1.423	1.407	101
Culture supernatant *E. coli* O113:H10	65,400	60,200	109

Evolution of ACC Comparability Studies

Large Comparability Study

Absorbance plate reader

rCR vs. Chromogenic LAL – ES
Standard curve 10 – 0.01 EU/mL
Spike: 0.1 EU/mL

Tube reader

rCR vs. Turbidimetric LAL – ES
Standard curve 1 – 0.001 EU/mL
Spike: 0.1 EU/mL

Evolution of ACC Comparability Studies

Commercially available media and buffers – plate-reader

| Sample | Dilutions | rCR | | LAL | | Relative Recovery % |
		Final conc. EU/mL	%PPC recovery	Final conc. EU/mL	%PPC recovery	
Mammalian Cell culture Media 1	1:4	< 0.04	117	< 0.04	175	
	1:8	< 0.08	117	< 0.08	156	
	1:16	< 0.16	120	< 0.16	161	N/A
Mammalian cell culture media 2	1:4	< 0.04	84	< 0.04	64	
	1:8	< 0.08	91	< 0.08	84	
	1:16	< 0.16	86	< 0.16	80	N/A
Insect cell culture media 1	1:5	< 0.05	97	< 0.05	87	
	1:10	< 0.10	102	< 0.10	103	
	1:20	< 0.20	104	< 0.20	107	N/A
Insect Cell culture media 2	1:5	< 0.05	147	< 0.05	138	
	1:10	< 0.10	122	< 0.10	114	
	1:20	< 0.20	117	< 0.20	105	N/A

Evolution of ACC Comparability Studies

Commercially available media and buffers – plate reader

Sample	Dilutions	rCR Final conc. EU/mL	%PPC recovery	LAL Final conc. EU/mL	%PPC recovery	Relative Recovery %
Cell culture supplement 1	1:10	< 0.10	87	< 0.10	82	
	1:50	< 0.50	91	< 0.50	92	
	1:250	< 2.5	89	< 2.5	89	N/A
Cell culture supplement 2	1:2	< 0.02	95	< 0.02	98	
	1:4	< 0.04	85	< 0.04	88	
	1:8	< 0.08	72	< 0.08	73	N/A
Cell culture supplement 3	1:10	< 0.10	92	< 0.10	93	
	1:100	< 1	91	< 1	92	
	1:1000	< 10	92	< 10	95	N/A
Cell culture antibiotic	1:10	< 0.10	91	< 0.10	95	
	1:100	< 1	89	< 1	87	
	1:1000	< 10	87	< 10	68	N/A

Evolution of ACC Comparability Studies

Commercially available media and buffers – plate reader

Sample	Dilutions	rCR		LAL		Relative Recovery %
		Final conc. EU/mL	%PPC recovery	Final conc. EU/mL	%PPC recovery	
Cell culture hormone	1:10	< 0.10	98	< 0.10	84	
	1:100	< 1	96	< 1	94	
	1:1000	< 10	99	< 10	100	N/A
Buffer 1	1:10	NR	32	NR	16	
	1:100	< 1	82	< 1	89	
	1:1000	< 10	85	< 10	97	N/A
Buffer 2	1:10	< 0.10	53	< 0.10	66	
	1:100	< 1	82	< 1	92	
	1:1000	< 10	88	< 10	92	N/A
Buffer 3	1:10	< 0.10	121	< 0.10	118	
	1:100	< 1	96	< 1	98	
	1:1000	< 10	91	< 10	93	N/A

Evolution of ACC Comparability Studies

Various water samples 1:50 (activated charcoal filtered water)

Plate reader

Sample	rCR Final conc. EU/mL	%PPC recovery	LAL Chrom Final conc. EU/mL	%PPC recovery	Relative Recovery % Mean rCR / Mean LAL *100%
Water #1	9.13	65	12.02	105	76
Water #2	5.74	101	4.33	115	133
Water #3	9.48	81	5.96	105	159
Water #4	6.21	100	8.35	101	74
Water #5	6.07	95	8.93	96	68
Water #6	4.67	95	4.63	105	101
Water #7	2.62	103	4.17	99	63
Water #8	2.56	65	2.09	105	122
Water #9	6.78	108	3.42	125	198

Tube reader

Sample	rCR Final conc. EU/mL	%PPC recovery	LAL Turb Final conc. EU/mL	%PPC recovery	Relative Recovery % Mean rCR / Mean LAL *100%
Water #1	10.34	59	8.78	61	118
Water #2	6.04	67	5.56	76	109
Water #3	8.27	62	8.58	62	96
Water #4	8.38	64	7.62	77	110
Water #5	13.55	70	7.06	75	191
Water #6	6.58	83	4.65	74	142
Water #7	3.78	88	2.63	89	144
Water #8	4.04	88	2.76	75	146
Water #9	8.16	137	5.38	81	152

Evolution of ACC Comparability Studies

Antibiotics and steroid – tube reader

Sample	Dilutions	rCR Final conc. EU/mL	%PPC recovery	LAL Final conc. EU/mL	%PPC recovery	Relative Recovery %
Atb 1	1:100	23.55	47	29.70	97	
	1:1000	13.44	91	23.63	116	50%
Atb 2	1:100	23.93	63	24.12	51	
	1:1000	21.89	106	25.82	113	92%
Steroid	1:100	< 0.1	90	< 0.1	127	
	1:1000	< 1	126	< 1	130	N/A

Evolution of ACC Comparability Studies

- **So far, all comparability results show an agreement between all rCR and LAL-ES**
  - Different reagents
  - Different instruments
  - Different sensitivities
- We continue to gather and accept samples from various users and sites
- We continue to support evaluation studies and assessments of rCR

What Is Next

What Is Next

- Publication in preparation on rCR next generation includes non-product specific method validation:
    - Linearity
    - Range
    - Limit of quantification
    - Accuracy
    - Precision
    - Specificity
- Assist end users with product specific method validations
    - Accuracy
    - Precision
    - Method suitability

What Is Next

- Continue with building the comparability library

- Provide the data to the pharmacopeias

- Internally, we will be validating the process water testing on rCR based on a completed assessment study

Conclusions

- rCR is well-developed and well-studied recombinant reagent for BET

- rCR is based on over a decade long history of development, experience and data

- rCR has been shown to report data comparable to LAL-ES

- The use of rCR is a single component of our approach to sustainable and reliable system

- LAL will continue to be used to ensure patient safety!